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THE BEHAVIOR OF MUSCLE MEMBRANE TREATED WITH A TRACE METAL CHELATING AGENT[1]

HOWARD JENERICK[2]
Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts

EIGHT FIGURES

Cupferron (a chelating agent for heavy metals) can block conduction in a mixed nerve (frog sciatic) without greatly increasing thresholds or depolarizing the injury potential (Jenerick, '57a). This seemed to rule out anesthesia or metabolic inhibition as the primary cause of the conduction block. One of the explanations offered at that time for these results was that cupferron inhibits the mechanism underlying the action potential. To test this hypothesis the blocking properties of cupferron were studied using single cells. The sartorius muscle fiber is eminently suitable for transmembrane stimulation and recording techniques. The present results suggest that cupferron interferes with the sodium current during the action potential in addition to inhibiting metabolism and depolarizing the resting potential by independent means.

METHODS

Sartorius muscle was obtained by dissection from *Rana pipiens*. The Ringer's fluid, aerated before use, was usually composed of 111 mM NaCl, 2.5 mM KCl, 4 mM $CaCl_2$, and

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[1] This investigation was supported in part by a research grant (B-861) from the National Institute of Neurological Diseases and Blindness and in part by a grant from the Muscular Dystrophy Associations of America.

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1 mM "tris" buffer, pH 7.2 to 7.4. Additions were made as required to this solution. All experiments were done at room temperature (20° to 25°C) unless otherwise noted. Electrical recordings were made with Ling-Gerard microelectrodes, a negative capacity preamplifier (the frequency response with an average 10 megohm electrode is D. C. to over 20 kc), and a dual channel oscilloscope. Stimulation was effected by a second microelectrode inserted into the desired cell near the recording site or by applying shocks across a partition in the chamber through which one end of the muscle was passed. In some cases the action potentials were recorded in the phase plane by displaying the original signal on the vertical axis of the oscilloscope tube and its first derivative on the horizontal axis. The phosphate fractions of muscle were analyzed with conventional colorimetric procedures. Further details appear in the text.

RESULTS

Resting potential. Measurements were made in 20 separate experiments on more than 400 fibers exposed to a Ringer's fluid containing cupferron at several concentrations from 3 to 20 mM. Generally speaking, cupferron caused a rapid fall in potential which terminated in about 20 minutes at a new level which in most cases was maintained for over an hour in the lower concentrations (3 to 10 mM). In higher concentrations (15 to 20 mM) the plateau was maintained in about half the experiments for a half hour (fig. 1). This plateau is relatively independent of concentration and the amount of depolarization seems to be independent of original resting potential. The membrane was depolarized by an average of 13 mv (range of 18 experiment averages, in 10 mM and higher, is 8 to 18 mv) from original levels ranging from 72 to 105 mv. If the muscle was returned to Ringer's fluid within a half hour or so, the resting potential began a slow return toward normal values. Otherwise, a slow decline at a rate proportional to concentration marked the end of the plateau period. The resting potential was usually depolarized to 50% of the control value by 20 mM cupferron in 2½ to three hours. Contracture was obvious after two hours when the potential reached 55 mv or less.

Many of these measurements were taken during experiments on propagating action potentials which were elicited by external fluid electrodes. Although fibers tested late in the experiment had been activated and contracted many more times than early ones, this did not accelerate or cause the depolarization. The depolarization rate was measured in another way with the same results. The mean injury (crush) potential of freshly dissected muscle is 46.7 ± 2.1 mv (9 muscles). Muscles placed in undisturbed beakers of Ringer's fluid with 20 mM cupferron lost half this potential in about 2½ hours. Except for the first hour, where the rate of diffusion to deeper fibers complicates the picture, the curves of potential as per cent of initial values against time are similar for both methods. It was also noted in this last series that high room temperatures (28°C) gradually depolarized the controls resting in Ringer's fluid by 6 to 7 mv during 4 to 6 hours. Lower temperatures (20° to 22°C) or the addition of 5 mM glucose (at 26°C) prevented or

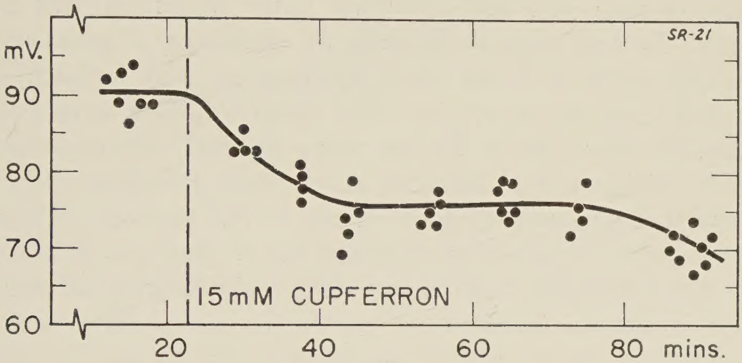

Fig. 1 The depolarization of the resting potential to a plateau near 75 mv. The muscle remained in a 2.5 mM KCl Ringer's fluid to which cupferron was added at 23 minutes.

lessened this decline. Cupferron-poisoned muscles were not protected by any of these procedures.

Action potential. If the resting potential is lowered by suitably raising the K^+ content of the Ringer's fluid, the spike height and hence the overshoot are also reduced. Block generally follows any further reduction of the resting potential below 55 mv when the spike height is very nearly 50 mv. On the other hand, if cupferron is present the spike height and overshoot decline more rapidly than resting potential, block occurs in fibers only moderately depolarized (near 75 mv), but the minimum spike height is still about 50 mv. In one experiment, out of 13 fibers, 6 with resting levels from 78 to 76 mv were conducting, with spike heights ranging from 49 to 67 mv. The other 7 fibers with resting potentials from 75 to 73 mv were blocked. Conduction returned within a few minutes after plain Ringer's fluid was readmitted, suggesting some effect at the surface. The next 15 fibers were conducting although the resting potentials were still at a low level, 75 to 67 mv. The spike heights of this last group increased to expected values for the resting potential at that time and then both rose back gradually to the original control values.

By least squares methods a second degree equation was fitted to the control (K^+ modified) data; $(\text{overshoot}) = 1.25\,(\text{spike height}) - 0.0043\,(\text{spike height})^2 - 57$, all values in mv. The data were obtained from 109 fibers with spikes ranging from 52 to 141 mv (fig. 2a). (The equation is not meant to imply that the relation is second order.) Similar calculations on data from 84 cupferron-treated fibers gave the following relation, also plotted in the figure: $(\text{overshoot}) = 2\,(\text{spike height}) - 0.0072\,(\text{spike height})^2 - 110$. From these expressions figure 2b was constructed. Ordinarily the scatter of data and the sharp fall of the overshoot obscure this action of cupferron. The question remained whether other parameters of the action potential could be altered.

The propagating action potential of nerve (Rosenblueth, Wiener, Pitts, and Garcia Ramos, '48) and of muscle (Tasaki and Hagiwara, '57; Jenerick, '57b) has several exponential portions whose rate constants are a convenient means of specifying the shape. In addition these constants indicate something of the membrane behavior and properties. For example, the rate constant of the action potential foot (k_r) is directly proportional to the square of the conduction veloc-

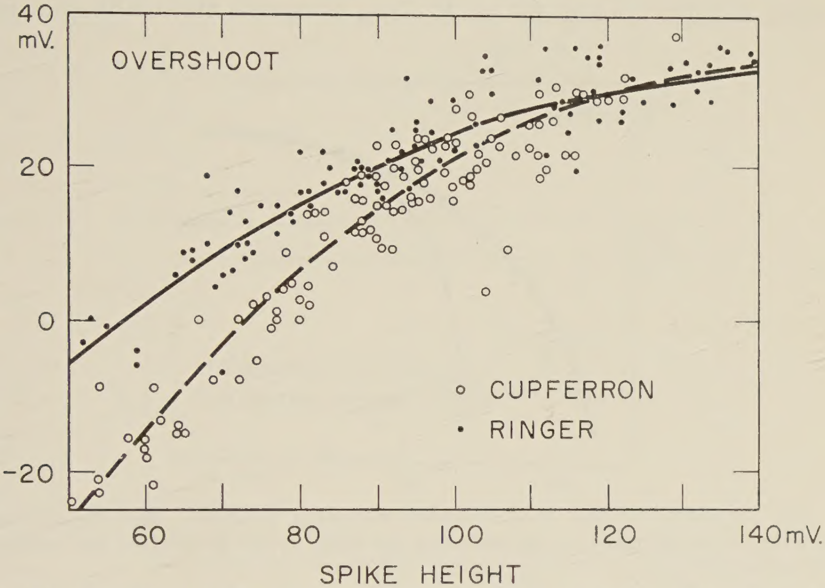

Fig. 2a The depression of the overshoot by cupferron. The solid circles refer to experiments in which the spike height was altered by an excess or deficit of KCl only. Cupferron was usually added to solutions supporting large to moderate spikes. As the compound lowered the resting potential the spike height fell more rapidly than in the controls. The curves are second order, fitted to the data by least squares methods.

ity. The negative afterpotential decays with a time constant very close, if not equal, to that of the membrane (Hagiwara and Watanabe, '55). These rate and time constants are easily obtained by recording in the phase plane where exponential processes appear as straight lines.

Such phase plane records showed that the rising and falling rate constants of an action potential are not altered by cupferron if the decreased spike height is taken into account (fig. 3). Because spikes are smaller in cupferron poisoned muscles, these parameters are reduced at any given resting potential. Although it is not too clearly brought out in the figure, which contains data from over 120 fibers, k_r and k_f are probably S-shaped functions of spike height. Recent experiments suggest that k_r may even decline with very large spike heights in low K^+ or K^+-free Ringer's fluid but this is a separate matter and is being studied further. Another variable showing an S-shaped dependence on spike height is the maximum rate of rise of the action potential. Again cupferron does not alter the dependence if the depressed spike height is accounted for.

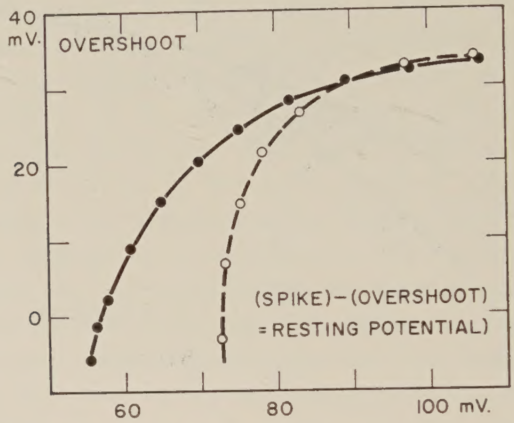

Fig. 2b The overshoot as a function of resting potential. This figure was obtained from the previous one by using the fitted curves to retrieve the resting potential data.

The first afterpotential (classically called the negative afterpotential) definitely represents a different process from the falling portions of the spike. The usual records of voltage against time show this poorly, if at all. One process leads smoothly into the other and the transition point is not easily distinguished. By recording in the phase plane where time does not appear, the two processes are clearly set off from each other by a pronounced break in the record (the time constants differ by 50-fold). The initial value of the afterpotential (measured at the point where the records break) is linearly related to the spike height by the following relation obtained from the data of 60 control fibers by least squares

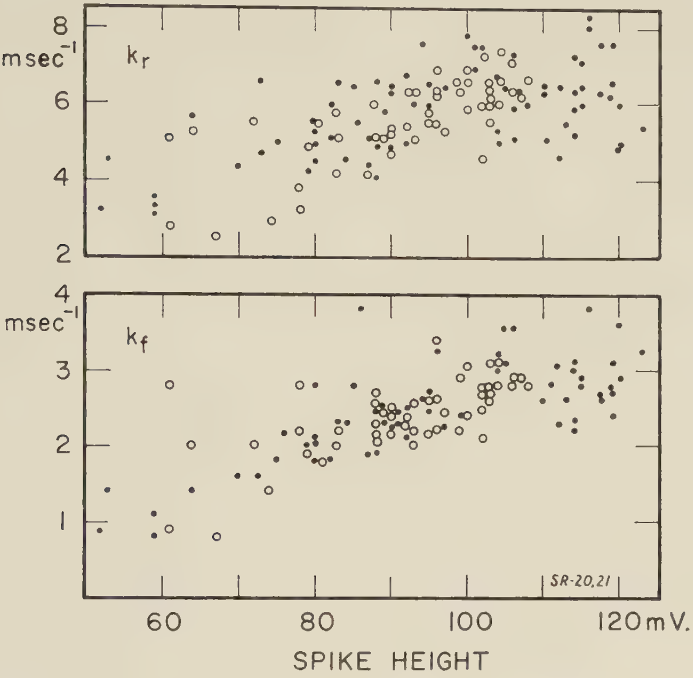

Fig. 3 The rate constants of the rising and falling phases of the action potential are unaltered if compared on the basis of the spike height. (Open circles plot cupferron data.) The rising phase refers to the exponential foot of the action potential up to the first inflection point. The falling phase is that portion between the second inflection point and the beginning portion of the first afterpotential. The similarity of these constants indicate that the spike shape is unaltered by cupferron.

methods; AP = 0.3 (spike height) − 6. The correlation coefficient is 0.81 (fig. 4). Over 350 measurements were taken in 26 experiments. Instead of plotting all of these, about 100 fibers of representative behavior were chosen for the figure. The variation of the afterpotential is not necessarily related to the KCl concentration. An occasional small or large spike was obtained in any given fluid yet the afterpotential was typical for that spike size. In cupferron the following differences appeared. The afterpotential was more rapidly reduced toward its final value than the spike height. In the figure, the first 25 measurements after cupferron addition are plotted separately. Nearly 30% of these fall below the line bounding two standard errors of the estimate about the curve. All but one of the last 23 measurements are within the 95% level; 15 late measurements are within the 50% level. Recently, Frank ('57) investigated the relation between the afterpotential and the resting potential. When K⁺ was varied from 0.1 to 10 mM the afterpotential shifted away from the expected locus. While this might be explained by a dependence on spike height as our figure 4 shows, matters are not that simple. If the Na⁺ concentration is altered, the afterpotential varies not with the spike height (table 1, Jenerick, '57b), but with the resting potential (Frank, '57).

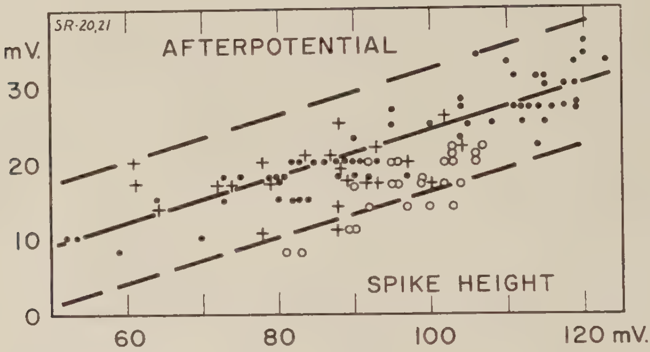

Fig. 4 The initial value of the first afterpotential as a function of spike height. The line of regression is fitted to the control data (variable K⁺ solid circles) and is bounded by lines subtending ± two standard errors of the estimate. The first group of measurements made after cupferron was added (open circles) tend to fall outside of the statistical limits, while those made toward the end of the experiments (crosses) are back within bounds. Inspection of the left hand portions indicate that the afterpotential may even be independent of spike height.

Electrical properties of the membrane. With the view that a study of several resting properties of the muscle membrane might furnish some clue to the mode of action of cupferron, the resistance and time constant were measured with square pulse techniques. It was found, however, that these membrane properties remained unchanged although the resting potential fell to 74 mv, (see table 1). The membrane resistance was previously shown to vary with the resting potential and K^+ content of the Ringer's fluid (Jenerick, '53). Fibers with the resting potential set at 75 mv with 5 mM K^+ would ordinarily have an $\overline{R}$ of only 2.5 to 2.7 × 10^5 ohms; the difference is therefore significant. Since the cupferron depolarized fibers were still bathed in a normal K^+ concentration, the possibility emerges that the external K^+ tends to set the membrane resistance.

TABLE 1

FLUID	NO. OF FIBERS	RESTING POTENTIAL	MEMBRANE TIME CONSTANT	MEMBRANE RESISTANCE
		mv	*msec.*	*$\overline{R}$, 10^5 ohms*
Ringer (2.5 mM K^+)	8	91.1 ± .14	23.7 ± 1.2	3.9 ± .3
Above + 15 mM cupferron	12	74.4 ± .15	21.5 ± 1.1	4.0 ± .2

The averages and standard error of the mean (corrected for sample size) are given in this table. $\overline{R}$ is the slope at the origin of a plot of membrane voltage against polarizing current.

Membrane local response. Two adjacent microelectrodes were arranged for simultaneous constant current stimulation and recording. Weak currents cause electrotonic potentials which are fitted by the error function given by Hodgkin and Rushton ('46). As the current strength is increased, the electrotonic potential shows a late occurring creep in a depolarizing direction. As the threshold is approached the creep is more rapid, occurs earlier in time, and may lead into a spike. However, since stronger shocks are used, the membrane reaches any given potential at an earlier time, and when series of increasing shocks were applied to the same fiber, the upward creep tended to occur at the same potential. This potential lies between 60 and 70 mv and depends slightly on the original resting potential which varied from 68 to 100 mv. Over 180 responses from 35 fibers have been analyzed. The onset of the upward creep was identified as follows. Since small electrotonic potentials follow the error function, one was selected as a ''predicter.'' Its ordinates were multiplied by the ratio of the current strengths of the responses to be tested to that of the ''predicter.'' These new curves are plotted with the actual records from the experiment and the discrepancies (Δv) are noted. When these are plotted as a function of the actual membrane voltage, as in figure 5, it is possible by extrapolation to determine rather closely the moment and potential when the local response begins. As long as the membrane remains in the resting state (where its characteristics are those of the model chosen by Hodgkin and Rushton) the discrepancies (or Δv) are zero as the first response in the figure shows. When the potential is reached where Na^+ conductance begins its increase, the potential is accelerated upward and the predicted curve is left behind. If activation does not occur until a certain potential is reached, the early portions of these curves should fit the error function as the figure does show. It is also worth mentioning that these responses are accelerating in depolarization even out to 100 msec. after the shock is started as if Na-inactivation or K-activation can be greatly delayed, are masked by a larger Na-activation, or come in at lower potentials. There is evidence in the figure which illustrates a finite time constant for Na-activation. Each succeeding Δv curve of a series is shifted toward lower voltages (larger depolarizations). Since the potential is moving more rapidly in time with stronger currents, any delay in the activation process after the activation potential is reached will allow a larger depolarization to develop before the upward creep is seen. In the presence of cupferron the upward deflection of Δv still appears around the 60 mv level. The activation potential may be shifted slightly downward, but slight inaccuracies in curve fitting and extrapolation do not allow a definite statement. In cupferron, the electrotonic potential is altered. The Δv is smaller, is delayed (note how successive curves are shifted to the right), and is usually terminated by an actual decay of potential below the expected value. If fresh Ringer's fluid is readmitted, the local response and spike activity returns but is shifted to earlier times.

If we can accept this kind of record as a rough indicator of the onset, rate, and magnitude of the membrane ionic conductance in the active and near-active state, we see that in muscle as in the squid giant axon (see below) cupferron in-

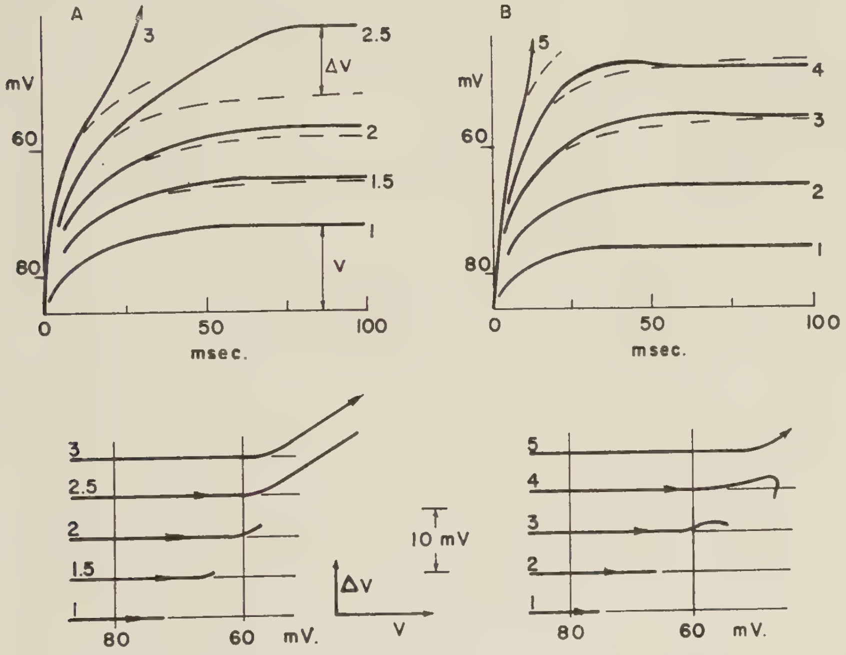

Fig. 5 Electrotonic potentials in Ringer's fluid (A) and 15 mM cupferron in Ringer's fluid (B). In the upper plots the solid lines are tracings from actual records, the dashed lines are the error function curves predicted by the lowermost record in each plot. The numbers next to each curve are the current strengths, relative to the lowermost record. The lower plots show the magnitude of the departure from the error function as a function of membrane voltage. Cupferron (lower right) depresses local activity, presumably by decreasing the sodium current.

terferes with the sodium current. This interference if great enough can account for the decline of the spike height and eventual block.

Through the kindness of Mr. John Segal the following information was obtained. Cupferron caused a 50% reduction of the inward Na current of the voltage clamped squid giant axon. The maintained K current was unaffected. The interference with the sodium current was almost completely reversed by washing with fresh sea water (fig. 6). The press of time unfortunately precluded further experiments.

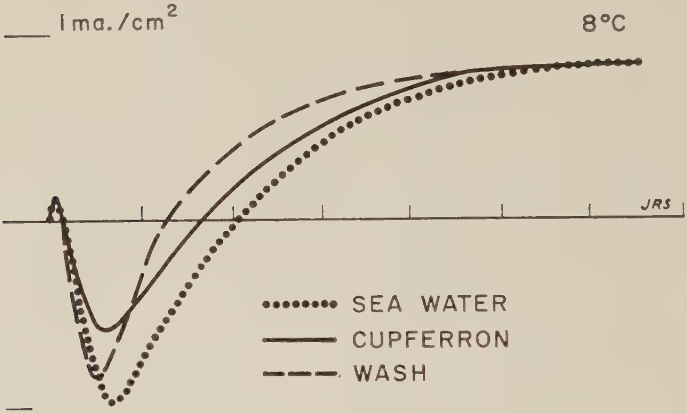

Fig. 6 The membrane currents of the voltage clamped squid axon before, during, and after the application of 10 mM cupferron. The steady state K^+ conductance is hardly affected while the changes in Na^+ following the chelater is also striking but unexplained. (Marks on abscissa are 1 msec.)

Sodium ion and cupferron. According to the Hodgkin-Huxley equations the sodium current is dependent on a number of parameters including the sodium potential. Does cupferron lower the overshoot in frog sartorius and current in squid axon by lowering the sodium potential? The answer is almost a categorical "no," because of the following lines of evidence: (1) When the sodium potential and the overshoot is reduced by replacing ½ of the external NaCl by choline chloride, the resting potential is increased (not decreased as by cupferron). (2) The concentrations of cupferron use here fall shy by more than 50% of that necessary (on a one to one basis) to remove sufficient Na ion from solution by chemical action. (3) There is no observable interaction between Na and cupferron. The conductance of 20 mM cupferron in Ringer's fluid was measured and found to be within 3% of the sum of the conductances of each solution. If Na were bound, the conductance of the mixture would fall because H ion is not released during the formation of cupferron complexes. (The 3% discrepancy could be explained by a one centigrade degree shift in temperature.) The evidence suggesting that internal Na concentrations are not increased is treated in the Discussion.

TABLE 2

FLUIDS	SLOPE, SPIKE HEIGHT	SLOPE, CRITICAL PREPOTENTIAL
2.5 mM K^+ Ringer	— 31 ± 2.4	11 ± 1
(RP range 80 to 95 mv)	(51)	(29)
Above + 15 to 20 mM cupferron	— 98 ± 6.2	61 ± 4.5
(RP range 75 to 86 mv)	(10)	(10)

Spike height and critical prepotential are functions of the log of the latent period. Therefore, an equation linking potential and time is of the following form; $V = K \pm k \log t$, where V is the spike or prepotential, K is an additive constant, and k is the slope. The table lists for multiple responses, the average k, whose units are mv, and its standard error.

Critical potential. An obvious consequence of the diminished local response should be an altered set of excitation parameters. The critical prepotential (P^*) of sartorius is a function of the resting potential and the latent period (t^*) (Jenerick, '56). These are in turn functions of other variables including the inorganic ions of the surrounding fluids. The previous paper reported mainly on the variations of single responses with varying stimulus strengths. In several instances multiple responses were seen, but the P^*-t^* function of these records was very similar to the same plot made from single responses (see fig. 5, Jenerick, '56). It now appears that this is not true for all conditions. Cupferron causes the P* to rise and the spike height to fall more rapidly with t* in multiple responses compared with a family of single responses (table 2). For example, in cupferron the P* for a single response occurring 10 to 15 msec. after the initiation of the stimulus in the range of 45 to 50 mv. If a stronger shock is applied such that the second spike of a train occurs at 10 to 15 msec. the P* will have risen to 60 mv (fig. 7). It will be noticed that no propagated responses are found beyond 20 msec.

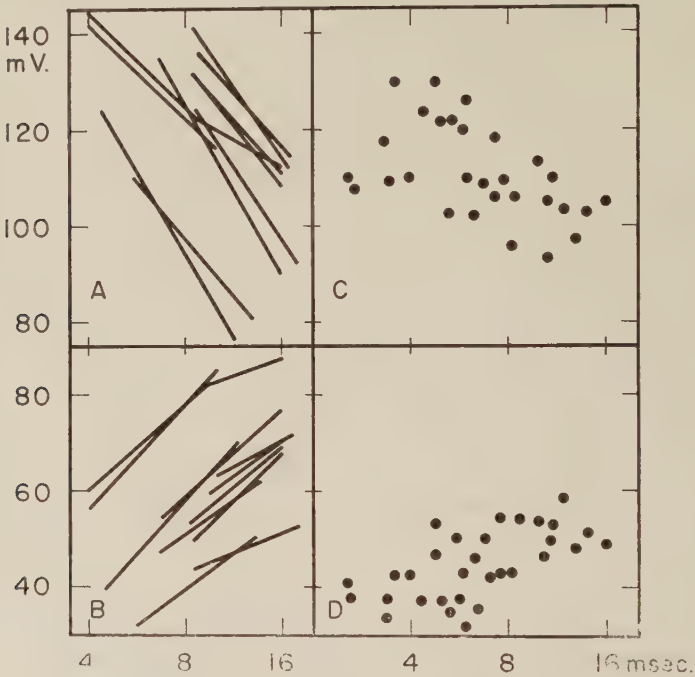

Fig. 7 The excitation and response characteristics (local stimulation) in cupferron treated cells. Critical prepotentials are plotted in B and D while spike heights are shown in A and C. Plots A and B are from experiments where multiple responses were seen. The straight lines connect the data obtained when two or three spikes occurred during the shock. In C and D only single responses at the indicated times were obtained. While these are very comparable to similar data from control fibers, the multiple responses show a much greater time dependence.

The P^* magnitudes are not great enough to disclose a marked rise of threshold. They are slightly larger than the P^* found in fibers depolarized to these levels with K, but this alone cannot cause the block. Fibers conduct in the presence of 20 mM Ca where the P^* can be higher and the resting potential lower (with appropriate amounts of K added to the high Ca solutions).

Metabolic effects. Although the oxygen consumption of the frog sartorius, the rat diaphragm, and rat heart cytochrome oxidase is not inhibited by cupferron at concentrations up to 20 mM/l (unpublished observations) the creatine phosphate stores are depleted (fig. 8). Because there is a variation from muscle to muscle, this figure was prepared as follows. In each experiment we determined for the control and treated muscle what percentage of the total phosphate was represented by each of the separate fractions. The percentages from the cupferron-treated muscles of one leg were then compared to the corresponding percentages from the control muscles from the other leg. This final percentage is plotted in figure 8 where the loss of creatine phosphate and appearance of inorganic phosphate are to be seen. The Δ7 phosphate (mainly ATP and ADP) is maintained, probably by the action of creatine phosphokinase. It has not been established yet if cupferron uncouples oxidation and phosphorylation or has some other more complex action. The actual phosphate concentrations are given in table 3.

The results of these experiments raised the possibility that the depolarization of the resting potential represented the loss of the "A" potential (Ling and Gerard, '49), which is directly proportional to the CrP concentration. However, the time course of the initial depolarization is much faster than the disappearance of CrP. The "A" potential of frog sartorius is approximately 25 mv. A depolarization of one half this magnitude occurred in a half hour while three to 4 hours were needed to cut the resting CrP level in half.

Ammonium ion. Cupferron is the ammonium salt of nitrosophenylhydroxylamine. Ammonium sulfate and ammonium chloride at concentrations up to 20 mM NH_4^+/l caused only negligible shifts in the electrophysiological and metabolic studies. It is clear therefore that the anion (the chelating portion of cupferron) is responsible for our experimental observations.

The cupferron complex (?). Generally speaking, the absorption spectrum of a chelating agent is modified when a metal is bound (Martell and Calvin, '52). Cupferron in Ring-

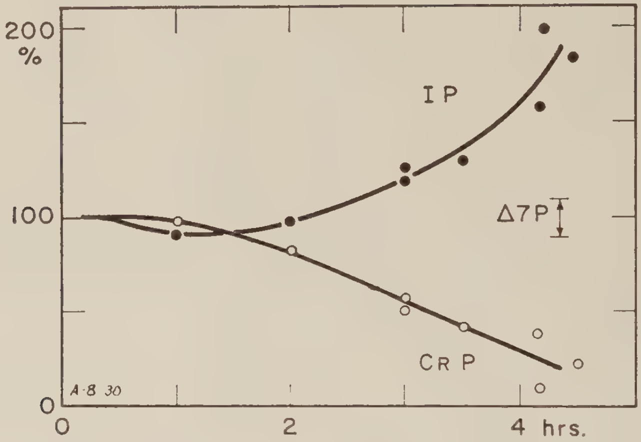

Fig. 8 Several phosphate fractions of muscle as a function of time in cupferron solutions. The fractions are compared to the values found in control muscle resting in Ringer's fluid for the same length of time. CrP is creatine phosphate. IP is inorganic phosphate. Δ7P is mostly ATP and ADP phosphate; which did not vary beyond the indicated limits.

TABLE 3

The phosphate fractions of frog muscle

MUSCLE TREATMENT	TOTAL PHOSPHATE	INORGANIC PHOSPHATE	CREATINE PHOSPHATE	Δ 7 PHOSPHATE
Ringer (control) (14)	1390 ± 35	320 ± 25	470 ± 24	270 ± 9
Ringer + 20 mM cupferron for 180–260 minutes (5)	1290 ± 22	420 ± 31	160 ± 20	250 ± 10

The unit of concentration is μg Phosphorous/gram muscle wet weight.

The figures represent the average and one standard error of the mean. The number of analyses is in parentheses.

er's fluid has an absorption peak at 283 mμ and a minimum at 245 mμ. If a muscle is soaked for about 30 minutes in Ringer's fluid with cupferron, the absorption spectrum of the fluid is altered. The former absorption peak at 283 mμ is decreased in magnitude and definitely shifted to 281 mμ while the former minimum is increased in magnitude and shifted slightly to a shorter wavelength. The responsible material is leached from muscle by plain Ringer's fluid. If cupferron is added after the muscle is removed, the same shifts occur. Fresh fluids and fluids in which muscles have been soaked have negligible absorption at these wavelengths. This material has not been identified nor has its physiological role been assessed. The absorption spectra of mixtures of cupferron and various heavy metals were measured in an attempt to identify the substance. The spectral shift is a function of both the metal and its concentration so that identification of the muscle substance at this time is not feasible.

DISCUSSION

Conduction block must occur when the action potential occupying a stretch of membrane is incapable of stimulating the resting membrane ahead. It was repeatedly observed during the course of these experiments that the smallest conducted spikes are about 50 mv, whether cupferron or excess KCl is present. Since the spike shape is similar under both conditions, the physiological stimulus is the same at this point of incipient block. Therefore, the membrane excitability at this time must also be similar. If so, the cupferron block is not primarily a result of an increased threshold. (There is a slight rise of critical prepotential but not above the control values (fig. 7). It seems necessary to suppose that the depression of spike height is the primary factor. What processes are responsible for this?

Ling and Gerard ('49) observed a decline of resting potential and phosphocreatine stores in muscles poisoned with iodoacetate and cyanide. Because the phosphocreatine stores are similarly depleted by cupferron it is clear that energy production has been stopped, and the late depolarization of the resting potential must be a consequence of this. The early, rapid fall to a plateau is reminiscent of the action of azide, but not of the usual inhibitors. Apparently the resting sodium conductance of the membrane has been slightly increased setting the potential at a level intermediate between the sodium and potassium potentials. This could lead to a net entry of sodium if the pump does not keep pace which would lower the sodium potential and the overshoot. It is true that the overshoot declines but it is not certain that the postulated events have occurred. As soon as the cupferron is washed out, the overshoot increases significantly before any upward drift of resting potential (which may in some cases continue to fall for a time). It is difficult to imagine how sodium is extruded without a simultaneous increase of the resting potential. Measurements of cell sodium concentration in 8 pairs of muscles were made with a flame photometer. Although the control values were a bit large (the frog colony suffered a parasitic infection at that time) there was no consistent increase of cell sodium concentrations in the muscles poisoned with 20 mM cupferron for several hours.

If the sodium potential has not been shifted then the sodium conductance (during the spike) must be suspected. The shape of the spike potential is not altered by cupferron if compared to normal spikes of similar magnitude, but we do not know how violent the change in the conductance must be to cause a noticeable alteration of the spike shape. Several observations point to an altered reactivity of the membrane in the presence of the chelating agent.

In particular, the behavior of the membrane to step current stimuli gives us further insight into the action of cupferron on membrane processes. Repetitive firing is infrequent while second and third spikes of a train are markedly reduced in size (table 2). The latter must be caused by some effect lasting after the first spike of the train since up to 8 or 9 msec. may elapse between consecutive spikes. Single responses are not reduced as greatly (fig. 7) so it is not some function of time. Since single responses are not preceded by large, long lasting depolarizations we may suspect that the effect is voltage dependent. Assuming a mechanism similar to that for the squid axon, we might interpret this as sodium inactivation. Given analogous voltage and time dependencies the inactivation could last long after a spike and cause both a rise in the critical prepotential and a drop in the height of subsequent spikes. Is there any other evidence for this assumption?

A membrane with distributed resistance and capacitance when depolarized by step currents will follow an error function as given by Hodgkin and Rushton ('46) as long as its properties remain fixed. Knowing about the nature of the ionic conductances from the work on the squid giant axon, we are in a position to interpret any departures from such an error function. (1) Increasing depolarizations (positive Δv, fig. 5) are moving toward the sodium potential and must be caused by sodium activation. (2) Decreasing depolarizations (negative Δv) are moving toward the potassium potential and must be caused by potassium activation. (3) Sodium inactivation will reduce a positive Δv by turning off the sodium current.

As soon as cupferron was added to the bath, the electrotonic potential was altered in a manner suggesting an earlier sodium inactivation. (This effect of an inhibitory substance should not be too surprising — inactivation is a measure of unavailable sodium carriers or sites.) The Δv is smaller and decays in every record (35 have been analyzed). Compare the terminal phases of the lower plots in figure 5. A diminished activation might lead to a smaller Δv at any given membrane potential, but it should not cause a late decay. Only when larger depolarizations are used does Δv become negative, as if potassium activation was now appearing and coming in at a lower potential near 50 mv. Not shown in the figure are responses from blocked fibers obtained late in the experiment. No indication of a sodium activation was seen. The membrane electronic potential closely followed the error function beyond the usual activation potential (which lies between 60 and 70 mv) down to around 50 mv when the potential began to drop back toward the resting level. We assume the sodium activation was completely lost or prevented leaving only potassium activation.

SUMMARY

1. The resting potential is rather quickly depolarized 13 mv (range of experimental averages, 8–18 mv) by 10, 15, and 20 mM cupferron. The new potential level is maintained up to an hour before falling slowly to zero (as creatine phosphate disappears). (figs. 1 and 8)

2. The height of the spike potential during this time is reduced to below expected values. (fig. 2a and 2b)

3. The shape of the spike (with the exception of the afterpotential (fig. 4) as measured by several parameters (fig. 3) is not altered.

4. The critical prepotential of single responses or of the first spike in a train (obtained with long duration shocks) are within normal limits. Succeeding spikes in a train are much reduced in size and have very large critical pre-potentials (table 2, fig. 7).

5. The membrane resistance and time constant are not altered (table 1) but the electronic potential and local response are altered in a manner suggesting that sodium current is decreased. (figs. 5 and 6)

6. It is concluded that conduction block is a consequence of this.

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THE SUBTHRESHOLD RESPONSE OF THE SINGLE CRUSTACEAN MOTOR AXON [1]

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TWELVE FIGURES

INTRODUCTION

Crustacean motor axons may be classified into three groups according to the oscillatory nature of the all-or-none response (Hodgkin, '48; Wright and Coleman, '54). When subjected to long duration direct current stimulation almost all crustacean motor nerve fibers tend to fire repetitively but this response is more highly damped in certain fiber types than in others. For example, the opener fiber from the walking limb will fire trains of impulses indefinitely during direct current stimulation whereas under similar conditions more than one spike has never been elicited from the medial giant fiber. Although some light has been shed on certain aspects of these functional differences, for instance, the rather intimate association of oscillatory activity with potassium ion concentration in the medium bathing the fiber, (Wright, Coleman and Adelman, '55) the basic excitation mechanism underlying these differences remains obscure. What sort of trigger mechanism can in one fiber elicit an almost continuous train of impulses while in another evoke only one spike? To answer this question, it is necessary to know what happens, electrically, in the single nerve fiber during just subthreshold stimulation by direct current because this subthreshold response, hereafter, called the local or initial response must obviously be correlated with the trigger mechanism of conducted impulses. Indeed, it is highly probable that the local or initial response is the trigger mechanism. Although the effects of short duration subthreshold stimulation has been described (Hodgkin, '38), with the exception of the present investigation little attention has been given to subthreshold excitation by long enduring current. Thus, the next step in the study of single nerve fiber excitability was to investigate the trigger mechanism of the all-or-none response by careful examination of the properties of the subthreshold response of the single crustacean axon.

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METHODS

In order to obtain an accurate recording of the local response it is necessary to lead off from the immediate area of stimulation, the cathode. No matter what recording technique is used a large shock artifact is unavoidable. The method chosen was the same as used in previous excitability studies (Wright, Coleman and Adelman, '55). Lobster axons were mounted on three electrodes, the V-technique, and suspended in oil floating on physiological solution. The oil-water interface was used as an external stimulating and recording lead. This system does not allow for the quantitative measurement of electrical events across a specific area of fiber membrane as does the intra cellular electrode, but it does guarantee the maintenance of a nerve fiber in nearly normal condition for many hours after dissection.

After the fiber was mounted on the electrodes it was raised from physiological solution through an oil-water interface into oil floating on the solution so that only a small length of fiber, approximately the width of the electrode (0.25 mm) remained in solution. Stimulating and recording are effected in this small area, the cathode. Thirty single lobster nerve fibers including 11 opener, 6 fast closer and 4 slow closer fibers from the walking limb and 9 medial giant fibers from the esophageal commissures (Wright and Reuben, '58) were used. These fibers ranged in size from 60 μ (slow closer) to 145 μ (medial giant) and were maintained in excellent condition for many hours in Homarus physiological solution (Cole, '41). The fibers were directly connected through low impedance platinized platinum electrodes to the Grass S-4 stimulator and Dumont 333 oscilloscope, the high sensitivity of which eliminated the necessity of any pre-amplification.

RESULTS

Part I. The local response and the artifact

I. The local response

Typical subthreshold responses to long duration stimulation of the opener, (A, B), fast closer, (C), and medial giant fiber, (D), are shown in figure 1. The subthreshold response, left hand traces, is distinguishable as a small deflection superimposed on the deflection caused by the stimulus current, the shock artifact. The dashed lines have been drawn in to make the superimposed deflection more obvious. In spite of the artifact it was possible to detect three different subliminal local responses, each specific to the type of axon stimulated. The local response of the highly repetitive firing fiber (opener) was of such small voltage amplitude (1 mv. or less) as to be difficult to distinguish in many cases and it appeared to terminate only after a long time, 15 msec., or more as indicated by the arrow in the figure. Local responses elicited from the giant axons such as the fast closer or medial giant were of much shorter duration, 1 or 2 msec., and considerably greater voltage amplitude, 5–20 mv.

II. The artifact

It became immediately evident that electrical activity generated by the fiber itself might be distinguished from the applied stimulation current, the shock artifact, only with considerable difficulty. Therefore, at the very outset, electrical recordings from non-active nerve tissue as well as active nerve fibers in various conditions were obtained, analyzed, and com-

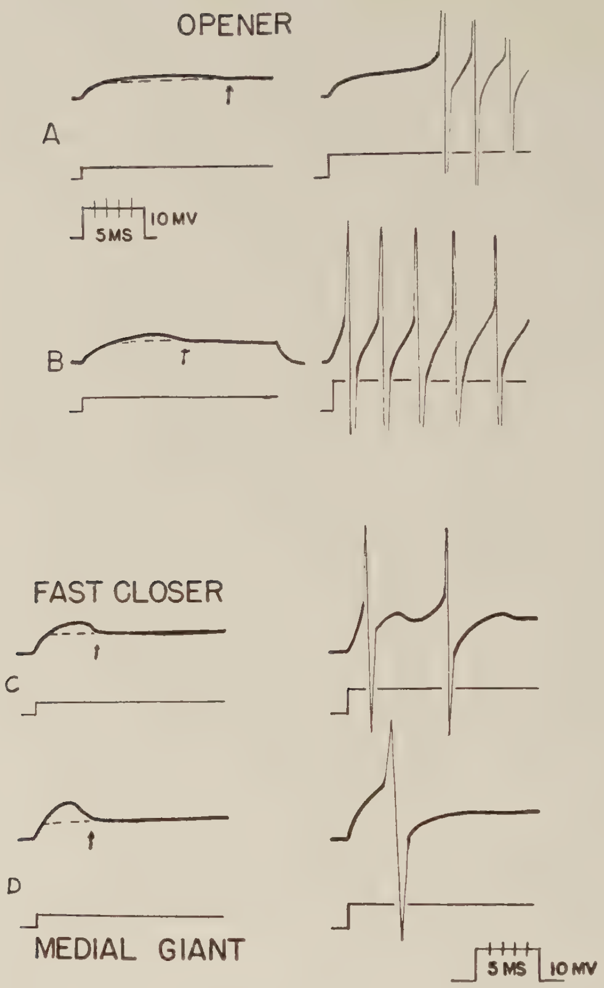

Fig. 1 Subthreshold and suprathreshold responses of three crustacean motor axons. A, Left trace shows the subthreshold response of an opener fiber superimposed on the shock artifact, the right trace shows the suprathreshold response. The DC stimulus is drawn in below illustrating relative strength of stimulation. B, Subthreshold and suprathreshold responses from the same fiber two hours later. The arrows indicate termination of subthreshold response. C, Subthreshold and suprathreshold responses from a fast closer fiber. D, Subthreshold and suprathreshold responses from a medial giant fiber. 5 msec., 10 mv. calibration (see text).

pared in order to have at first hand a clear picture of the difference between artifact and local response. Examples of such recordings from an excellent opener fiber preparation are illustrated in figure 2. Pictures A and E show the oscilloscope deflections, negative upwards, positive downwards, produced in two superimposed sweeps by a 10 msec. DC shock (A) and by a 0.1 msec. pulse (E) with the nerve fiber and electrodes totally immersed in physiological solution. The upper beam was connected directly to the stimulator leads, the lower beam to the recording leads on the fiber. In solution the upward (negative) and downward (positive) shock deflections were symmetrical and similar in shape in both beams. The deflection in the upper beam connected directly to the stimulator leads was blunted like the deflection in the lower beam because the low resistance of the medium acts as a shunt across the output of the stimulator. Pictures B and F illustrate the oscilloscope deflections with the fiber raised in oil. The upward or negative deflection was just threshold value with DC stimulation as evidenced by the appearance of a spike after considerable latency. The short duration pulse (F) was just subthreshold value. The dashed line is a trace of the downward or positive pulse inverted to show the difference in electrical response to negative (cathodal) and positive (anodal) stimulation. The negative response was always the larger of the two. This asymmetry, particularly noticeable in F, is, of course, due to active depolarization by the fiber itself at the cathode. Just as in figure 1, this response (B) was barely distinguishable with long duration pulses of just threshold or subthreshold amplitude when applied to a highly excitable axon. (Note dashed white lines.) When such a fiber was fatigued by continuous high frequency (100 cycles per second) stimulation or simply allowed to deteriorate (see fig. 4), recordings such as those shown in pictures C and G were obtained. A large local response appeared early on the DC shock artifact and the asymmetry of the short duration pulse deflections became noticeably increased. Again, the white dashed line is the trace of the positive pulse in-

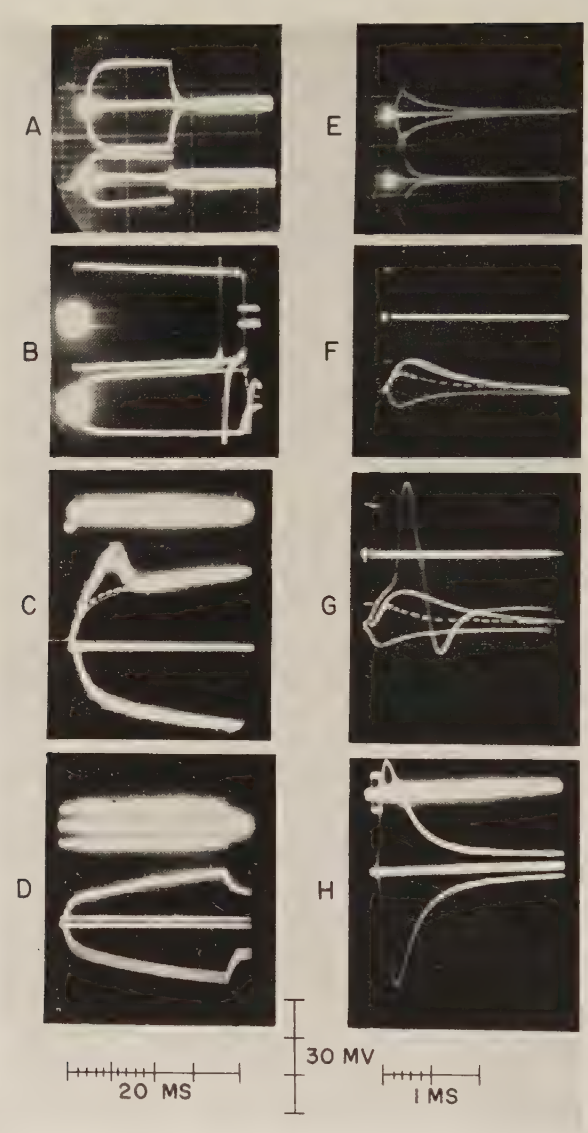

Fig. 2 The shock artifact and subthreshold or just threshold response from an opener fiber produced by long duration 10–20 msec. DC stimulation (left column) and short duration 0.1 msec. shock (right column). Pictures are time exposures of two or more sweeps on the oscilloscope screen. Pictures A and E were recorded from fiber immersed in physiological solution, B and F raised in oil, C and G after fatigue by 100 cycle/sec. frequency continuous stimulation for 10 minutes, and D and H after irreversible inactivation by high (250 v) voltage. Upward deflections negative (cathodal), downward positive (anodal).

verted. Recordings D and H were obtained from the same nerve fiber after permanent inactivation by applied high voltage (250 volts). The deflections negative and positive were absolutely symmetrical and no superimposed deflections, only the artifacts, were recorded. The time constant for the nerve fiber was reduced in value (see fig. 4) also by this severe treatment.

III. Difference between artifact and local response

A simple analysis of these recordings is shown in figure 3. Tracings of the oscilloscope deflections due to long (upper traces) and short (middle and lower traces) duration shocks are drawn in solid lines. Curves given by the equations of an electric circuit containing a resistance and capacity in parallel are indicated by the circles. In A and B these equations were plotted using the value 0.8 msec. for the time constant RC, in C the value was 0.4 msec. A small portion of the trace in A deviates from the calculated curve forming a hump. The area (1) under this hump indicates the local response from a freshly dissected highly repetitive responding nerve fiber. A trace of this response in the fatigued fiber or non-oscillatory type is indicated by the shaded area under the dashed line (2) near the start of the curve. There was good agreement between the recorded artifact curves and the graphical expression of the equations. The equations apparently describe the large cathodal deflection shown in B (solid circles) but only if it is assumed that the depolarization behaves as if the stimulus were applied for 0.2 msec. instead of 0.1 msec. In other words the nerve fiber continues generating current after the stimulus is turned off. The solid circles are a plot of the same equations $RC = 0.8$ msec. except a higher voltage value E_∞ has been used and assumed to last 0.2 msec. The area between these two curves represents the local response generated by nerve activity because the anodal response is considered a passive one.

In figure 3C is shown the tracing of the recording taken from a dead fiber (fig. 2H). The upward and downward deflections

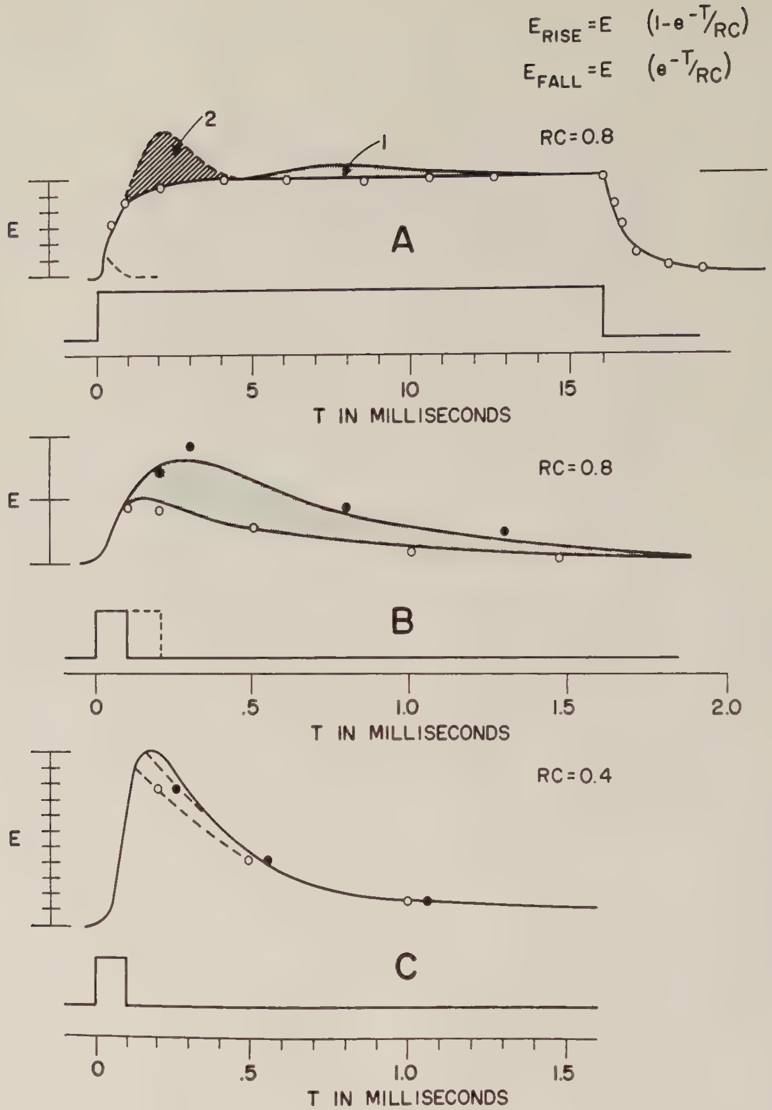

Fig. 3 Analysis of records from figure 2 to distinguish artifact from nerve activity. The upper curves are traces of deflection recorded in figure 2B and C caused by long duration stimulation. The open circles are points calculated from the equations with RC = 0.8 msec. The small dashed line is a trace of curves produced by short duration stimulation shown at faster sweep speed in the middle and lower drawings. The middle traces are taken from figure 2F and show cathodal and inverted anodal deflections produced by 0.1 msec. shock. Open circles are calculated points with RC = 0.8 msec. The closed circles are calculated points, RC = 0.8 msec., but plotted for a stimulation duration of 0.2 msec. instead of 0.1 msec. as shown by dashed square wave trace. Lower trace from figure 2G shows deflection by inactivated fiber. Dashed lines show the deviation of calculated curves from deflection tracing assuming rise time was same as shock, 0.1 msec., open circles, or slightly longer closed circles (see text).

were symmetrical, indicating no depolarization other than the shock artifact. A good fit by the calculated curves was obtained using the value 0.4 msec. for RC. Probably the resistance of the RC circuit had been decreased with inactivation causing this time constant change.

IV. Effect of fatigue or deterioration

Deterioration of a single nerve fiber with time after isolation, or with fatigue from continuous stimulation is evidenced by a rise in threshold of excitability, a loss of the oscillatory nature of the all-or-none response (Wright and Coleman, '54; Wright, Coleman and Adelman, '55) and a change in the shape of the local response. The voltage amplitude of the local response is increased and the duration reduced in value. An extremely subtle example of deterioration may be seen by comparing figure 1A with 1B. After three hours in solution the duration is considerably less in B than in A as indicated by the position of the arrow and the voltage has increased to an amplitude large enough to make the response more clearly discernible.

A striking example of the effect of fatigue by the continuous stimulation of a fiber raised in oil on both the local and the all-or-none response is shown in figure 4. At time zero (A) the spike deflection is large in the multiple trace recording while the upward subthreshold deflection is only slightly larger than the downward one. The asymmetry of these local response deflections becomes more pronounced in B after 5 minutes of stimulation and especially noticeable in C after 10 minutes. It is of extreme interest to note that concurrent with the local response increase there is a very definite decrease in amplitude of the spike. It would appear that a portion of depolarization normally associated with the spike in a fresh axon has been shifted to the local response by fatigue. Oscillatory activity with DC stimulation was abolished and the response to subthreshold DC stimulation (see fig. 2C) closely resembled that obtained from the normally non-oscillatory responding giant fibers (fig. 1C, D). Thus, the local response of a highly excitable (low threshold) repetitive firing fiber changed with deterioration or fatigue to become similar, if not identical, to the local response of a normal freshly dissected giant fiber of the non-oscillatory responding class.

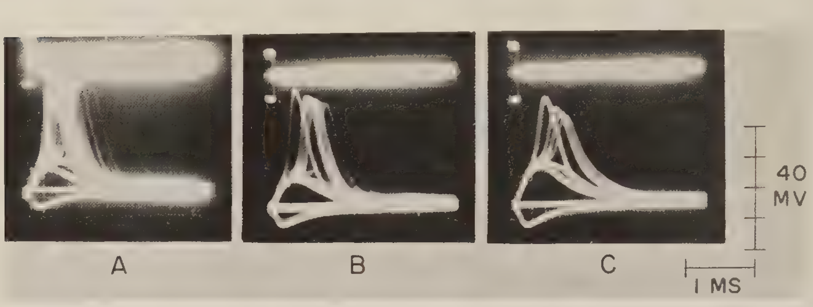

Fig. 4 Effect of fatigue by continous stimulation of an opener fiber raised in oil. Multiple trace recordings show spikes triggered by local responses, negative upward deflections, and passive anodal response, downward deflection, at the start of stimulation A, after 5 minutes B and 10 minutes C (see text).

Part II. The action of ions and chemical agents on the local response

I. Effect of excess potassium

The loss of the oscillatory response to direct current stimulation in a deteriorating or fatiguing fiber is evidence of a rapid increase in accommodation, probably caused by the loss of potassium from the interior of the fiber to the surrounding medium, thereby decreasing the potassium concentration gradient between inside and out. This has been shown to be the case experimentally by simply adding small amounts of potassium to the physiological solution bathing a fiber, (Wright, Coleman and Adelman, '58). The first step in this study, then, was to investigate the effect of excess potassium on the subthreshold response, for if the contour of the local response is to be associated with the oscillatory nature of the all-or-none response the experimental change of the oscillatory response should result in a change in the contour of the local response.

Typical recordings of the effect of two times the normal concentration of KCl on an opener fiber are shown in figure 5.

Normally subthreshold stimulation produced a local response of long duration and low voltage, threshold stimulation, a long latency before firing, and suprathreshold stimulation, an oscillatory all-or-none response (upper traces, left to right). After immersion for but a few seconds in excess potassium solution the subthreshold response was increased in amplitude and decreased in duration as shown by the two traces (middle on left) which were obtained 30 seconds apart. At the same time the latency to threshold stimulation was reduced and repetitive firing disappeared, (middle traces). Reimmersion in normal solution brought about reversal of this potassium effect, the beginnings of which are shown in the lower traces. Often during this recovery period the response to threshold stimulation was a sharply peaked local response (middle lower trace), which may include a small spurious spike (Hodgkin,

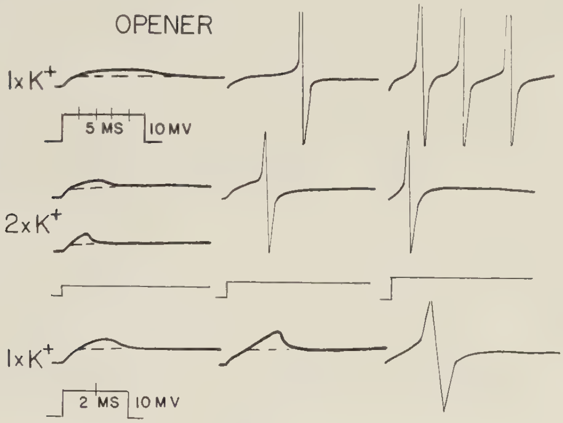

Fig. 5 The effect of excess potassium on subthreshold, threshold and suprathreshold responses of an opener fiber, left to right. Upper row of traces show the recordings obtained from a freshly dissected axon. Middle row of traces obtained after brief immersion in 2 × normal potassium concentration solution. The two left-hand traces in the middle row show the subthreshold deflection recorded after 10 seconds (upper) and 30 seconds (lower) immersion. The bottom row of traces are of recordings from the fiber in normal solution again. Shock tracings are included to show the relative strength of stimulation; 5 msec. and 2 msec., 10 mv calibration.

'38). The responses of this fiber became similar to the traces in the top row and reversal of the potassium effect was complete after one hour recovery time. As predicted excess potassium concentration did cause the contour of local response deflection to change to resemble the local response of a non-oscillatory responding fiber.

II. "Turn-off" of the subthreshold response

It is important to draw attention to the fact that a local response initiated by direct current stimulation does not last indefinitely but has a finite duration, or in other words, it "turns-off." In this respect the subthreshold response resembles the all-or-none spike. Deterioration, fatigue, or excess potassium produce a more rapid decline in the local voltage and this change in the contour of the local response parallels the increase in accommodation observed in excitability curves obtained under similar conditions (Wright, Coleman and Adelman, '55). The next step then was to compare the shape of excitability curves obtained by the dual shock method (Wright, Coleman and Adelman, '55) with the contour of a subthreshold response.

III. Excitability curves versus subliminal response

Subliminal responses from a medial giant fiber are shown in the upper part of figure 6. The fiber was stimulated continuously at 5 cycles/sec., and trace 1 was obtained at the beginning of stimulation; trace 2 after three minutes; and trace 3 after 10 minutes. The lower three curves are excitability curves obtained by the dual-shock method (Wright, Coleman and Adelman, '55) from the same medial giant fiber during the same 10 minutes of continous stimulation. These excitability curves, inverted so as to be more easily compared with the local response traces in the upper part of the figure, are strikingly similar in shape to the subthreshold response deflections. Indeed, changes with time, in both sets of curves are almost identical. For example, the local response duration decreased in 5 minutes from 9 msec. to about 7 msec., or 23%.

During the same period of time the high excitability phase had decreased from 7 msec. to 5 msec., or about 28%. After 10 minutes the local response had decreased to 5 msec. or an additional 28%, and the hyperexcitability phase had decreased to 4 msec., 20% more. Clearly with continual stimulation, the per cent changes in duration of both local response and hyperexcitability during subthreshold stimulation were remarkably similar. Although the shapes of the voltage trace and the excitability curve were not identical, they were similar enough to conclude that a definite correlation between local response and the excitability curve exists and the implication here is that excitability curves obtained by the dual shock technique are actually graphical expressions of the contour of depolarization produced by subthreshold DC stimulation. The fact that in highly repetitive firing fibers the excitability curve reaches its maximum value and remains there for a long period of time just as the local response in such fibers appears to last almost indefinitely (Wright, Coleman and Adelman,

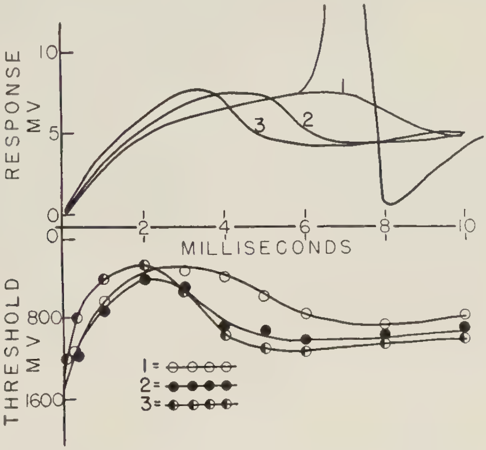

Fig. 6 The local subthreshold depolarization deflection, upper traces, and dual-shock excitability curves, lower traces, obtained at time zero, three minutes and 10 minutes during continuous 5 cycles/sec. stimulation of a medial giant axon. The excitability curves have been inverted to make comparison easier (see text).

'55, fig. 10), bears out this conclusion. As further proof, the modification of the excitability curves with excess potassium shown previously (Wright, Coleman and Adelman, '55) is almost identical to the change in shape of the local response curves illustrated here (fig. 5).

It has already been suggested in calling attention to the finite duration of the local response, that the response resembles the spike in that it "turns off." A true "turn-off" should require a recovery time or, in other words, produce a refractory period. The next step was to investigate the excitability of the fiber during and immediately following a local response initiated by DC stimulation.

IV. Refractoriness after the local response

Excitability during and following the local response was studied by employing an initial subthreshold DC shock and either subthreshold or just threshold short duration (0.1 msec.) shock at varying intervals of separation. Typical results obtained from a fast closer fiber are shown in figures 7A and B. In A the second shock was just subthreshold and produced a large local response deflection when the interval between it and initial response to subthreshold DC was large, (8 msec.). As the interval was reduced as shown by the multiple sweep trace the second response deflection was reduced to only the artifact. When the interval became so short that the second shock occurred near the peak of the initial response, summation took place and a spike appeared. The reduction of the local response initiated by the second pulse with progressively decreasing time intervals is an indication of refractoriness following the first depolarization.

This is more clearly shown in B where the scond shock was threshold and elicited a spike if the interval between it and the response to the DC pulse was 8 msec. Reduction of this interval caused the extinction of the spike and a decrease of the local response amplitude. When the interval was very short summation occurred and the spike reappeared.

These data show that a refractory period can exist following the turn-off of a local response set up by subthreshold DC stimulation. The question now arose: Since the local response apparently has more than one spike-like property was it, too, dependent upon ionic exchange such as the influx of sodium followed by the outflux of potassium? The next step then was to investigate the effect of sodium ions on the local response.

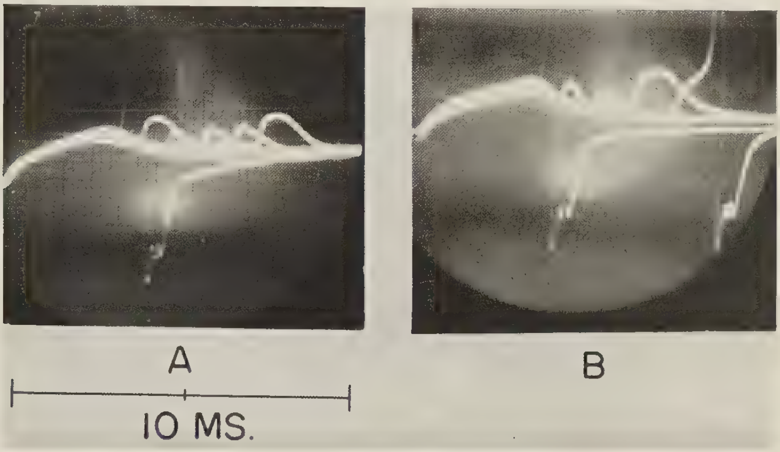

Fig. 7 Multiple sweep recording of a fast closer fiber local response produced by DC stimulation followed by the response to short duration (0.1 msec.) shock given at various intervals up to 8 msec. after the start of the DC. In A the second shock is just subthreshold, in B, just threshold when interval is 8 msec. or greater. Note the decrease of the second response amplitude with decrease in interval illustrating refractory period.

V. The effect of sodium on the subthreshold response

Single axons were immersed in solutions totally lacking in sodium. Isotonicity was maintained by substituting either dextrose or choline chloride for sodium chloride. No buffers were included, (Wright, '56a). Typical data from a fast closer fiber are shown in figure 8. The upper traces (A) show on the left the response to just threshold DC stimulation before immersion in NaCl-free solution, in the middle the response to strong DC 30 seconds after immersion in NaCl-free solution and on the right the threshold response after 10 seconds reimmersion in normal solution.

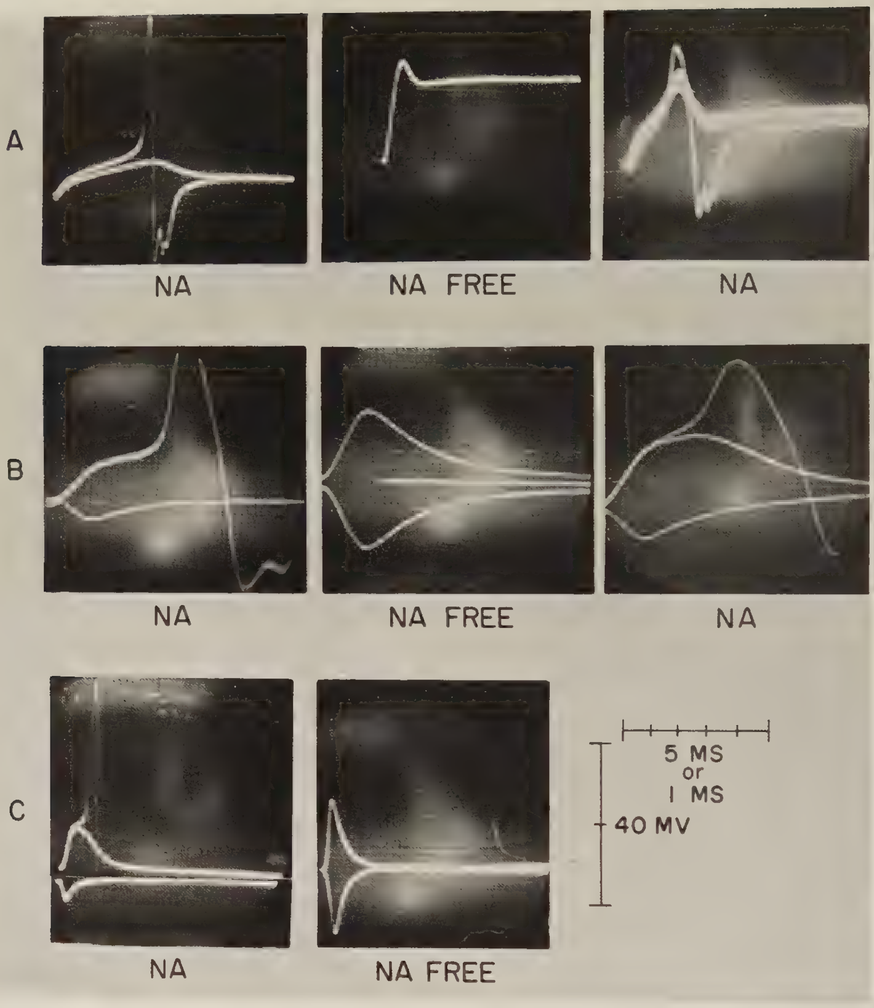

Fig. 8 Effect of a sodium free medium, choline chloride substituted, on the response of a single fiber to just threshold DC stimulation upper row of traces (A) and to short duration pulses, middle row of traces (B) at fast sweep, and lower row (C) at slow sweep speed. The pictures on the left were recorded from the fiber before immersion in NaCl-free solution, the middle pictures in each row 30 seconds after immersion in the NaCl-free solution and the right-hand picture, 30 seconds after reimmersion, in normal physiological solution. Double time scale 5 msec. for A and C, 1 msec for B. Note collapse of local response during falling phase of spike.

The effect of sodium-free solution, whether dextrose or choline chloride was substituted was the remarkably rapid extinction of all electrical signs of nerve activity. Both the spike and the local response disappeared instantly. Reappearance of these potentials with replacement of NaCl-free by normal physiological solution was equally dramatic.

The middle three traces (B) show the effect of sodium removal on the response to just threshold short duration (0.1 msec.) stimulus. On the left, the cathodal (upward) deflection is much larger than the anodal and elicits a large spike. In the middle, with removal of sodium the upward and downward deflections became symmetrical, no spike was evocable, indicating complete inactivation (see fig. 2H). On the right in the presence of sodium the asymmetry of the local responses returned and a smaller, broader spike was elicitable. The fact that it made no difference, the results were the same, whether chloride was included in the medium or not, pinpoints the source of electrical activity to the sodium ion alone.

In figure 8C are shown two recordings obtained with slow sweep speed from an axon before and immediately after immersion in NaCl-free solution. These responses were elicited by short duration cathodal and anodal shocks. The asymmetry of the local response deflections in particularly noticeable in A and also a faint spike trace. In B the deflections are perfectly symmetrical (compare with fig. 2H).

Surprisingly little sodium was needed to restore electrical activity to the membrane. After total inactivation by sodium-free solution, 10 seconds immersion of a fiber in only 10% normal sodium chloride solution brought on the return of the local and all-or-none response. Indeed, on several occasions during recovery in such solutions from exposure to sodium chloride-free solution an extra-ordinary response was obtained which is worth mentioning.

VI. The high-frequency oscillatory all-or-none response

Typical recordings of such a response are shown in figure 9. Immediately after immersion in a solution containing only

10% normal NaCl concentration weak and strong DC stimulation produced the recordings shown in upper pictures, left and right, respectively. Thirty seconds later with the appearance of local activity an oscillatory all-or-none response was evocable by DC stimulation which at near threshold voltage attained a frequency of about *1000* cycles per second. The response consisted of diphasic all-or-none spikes of 1 msec. duration appearing one right after another, with no intervening base line spacing as usually is recorded with repetitive firing, (fig. 1, upper right). Indeed, the sound of the oscillation through a loud speaker was a high whistle. Furthermore, it was only necessary to apply a stimulus of just above

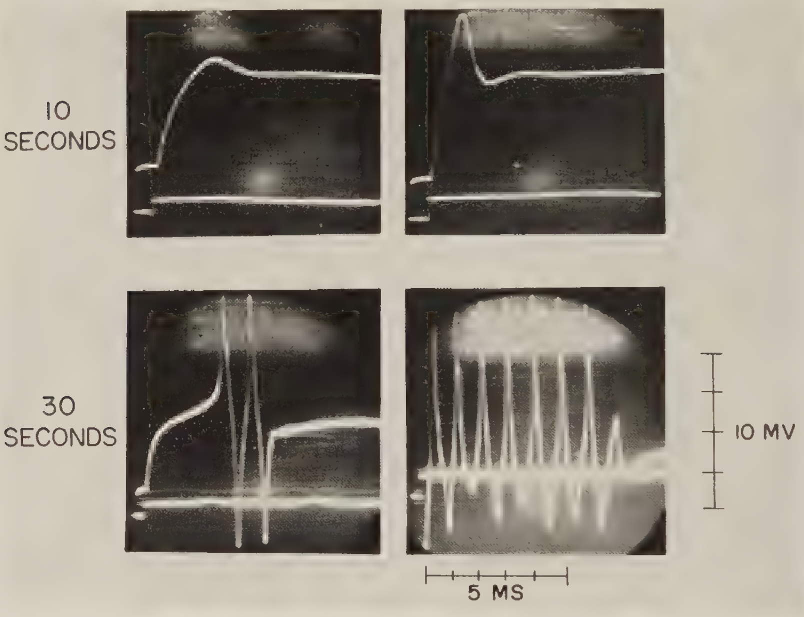

Fig. 9 Just threshold (left) and slightly suprathreshold (right) responses recorded from a fast closer fiber 10 seconds, (upper row), and 30 seconds, (lower row), after immersion in solution containing only 10% normal NaCl concentration (choline chloride substituted) after complete inactivation by NaCl-free solution. Note unusually high frequency (nearly 1000 cycles/sec.) after 30 seconds in this low sodium solution.

threshold strength to evoke this high frequency oscillation. The significance of such extraordinarily high frequency responses is not known, but similar recordings have been described as emanating spontaneously from certain areas of the CNS.[2]

VII. Is there a chemical mediator?

It has been suggested (Wright and Adelman; '56) that acetylcholine released in the membrane by the stimulating current initiates the permeability increase to ion flow and there precipitates the spike. It is implied by this suggestion that the presence of this agent is essential for the initial depolarization of the membrane, i.e., the local response. The final step was to consider the possibility of a chemical mediator between applied current and local response.

If a chemical mediator, acetylcholine, theory were correct, the local response should be markedly influenced by the amount of the agent present and the length of time it lasts. Thus, the local response should be very definitely prolonged by the application of an anticholinesterase, such as eserine. Assuming the amplitude of the response to be due almost entirely to sodium ion influx, eserine might not necessarily affect the local responses voltage.

The effects of eserine on the responses from an opener fiber are shown in figure 10. The spike was rapidly abolished by eserine but there was no significant change in the local response. Many fibers were subjected to the eserine treatment and all with the same results, abolition of the spike and no change in the local response.

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[2] The author is greatly indebted to Dr. Eldon Foltz for personally communicating the extremely interesting recent findings of Dr. Arthur A. Ward, Jr. and coworkers of similar remarkable high frequency repetitive responses emitted spontaneously by chronic cortical epileptic foci. These observations are described briefly in a paper entitled, Observations on single units in chronic cortical epileptic foci and in normal or strychninized cortex, appearing in Electroencephalography and Clinical Neurophysiology Journal, 1955, *7:* 478–480.

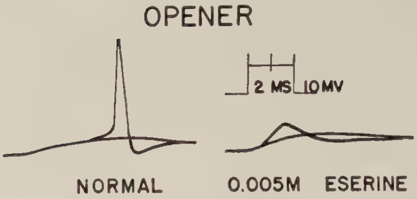

Fig. 10 Effect of eserine sulfate on the subthreshold and all-or-none response of an opener axon. On left, the original normal responses. Note collapse of the subthreshold response during the falling phase of the spike (see text). On right, the response obtained after a few minutes in 0.5% eserine solution. The subthreshold response was not changed, but the all-or-none response markedly reduced (see text).

DISCUSSION

The very existence of the subthreshold response is due to the sodium in the medium surrounding a nerve fiber. Without sodium in the medium no response of any kind is evocable from an axon. Even when the sodium current is turned off or inactivated during the falling phase of a spike (figs. 8 and 11) no response is recordable. This conclusion was predicted theoretically by the Hodgkin-Huxley axon model (Hodgkin and Huxley, '52) and experimentally by results obtained from crustacean axons subjected to very low temperature (Wright, '58, page 60). The chloride ion is ineffective and hence has nothing to do with the source of depolarizing current. Certain interesting properties of this sodium potential, the local response, need explanation, however. Why is the amount of depolarization and its duration during DC subthreshold stimulation dependent upon the type of axon stimulated or the condition of the fiber? For what reason does the local response of the opener fiber, an oscillatory responding type, consist of a long lasting low voltage while the local response of a medial giant fiber or the same opener fiber fatigued or deteriorated consists of short duration high voltage?

Some time ago it was suggested (Wright and Adelman, '56) that a chemical mediator released in the membrane by stimulation initiated the subthreshold depolarization. The shape and duration of this response was said to be directly related to the amount of substance released and the rapidity with which it was dissipated. In the light of the results of the eserine experiment, unless a mediator other than acetylcholine is to be postulated, this theory becomes untenable. The cholinesterase inhibitor did not affect the subthreshold response in any way. It is possible, though highly unlikely, that the effect of acetylcholine release is so small (Nastuk, '55) in the absence of sodium as to have escaped detection, but the indication is that the presence or absence of this agent has little influence on excitability. The effect of eserine on the all-or-none response, that is the blocking action, simply substantiates an argument put forward in another paper (Wright, '56b) that eserine, like other narcotics, somehow, inactivates the highly vulnerable all-or-none sodium release mechanism. Acetylcholine is not included in this argument either. The local response, like the all-or-none must be entirely due to ionic current and its properties must be explained on this basis.

Were we able to eliminate the shock artifact the deflections we would probably see are illustrated schematically in figure 11. The electrical response, local and all-or-none of the three fiber groups, oscillatory, slightly oscillatory, and non-oscillatory have been sketched in A, B, and C. The shock artifact is included as a light dashed line and the heavy double horizontal lines indicate threshold. In the case of the highly oscillatory responding fiber (A) the threshold is so low that it requires very little current to stimulate and as shown in the figure this membrane current, sodium influx, may actually produce a deflection of the same size or even smaller than the artifact itself and hence, not be observed except at regular intervals of repetitive firing. At these moments, crests of an apparent oscillation of the local response (Hodgkin and Huxley, '52) may appear above the artifact and become especially distinguishable, if for one reason or another, a spike is not elicited leaving a gap in the spike train (note fig. 1C). It is suggested that each crest or hump shown is a depolarization or potential caused by a sudden additional influx of sodium. The true local response observed in the highly excitable fiber may be just each little hump or crest, the rest of the deflection being artifact depolarization by the applied current. The apparent long duration of such a local response is explained by the long latency to the first crest. The slight indenture produced in the deflection by the falling phase of this little hump (see arrow, fig. 11B) makes is appear as if a local response has lasted a great length of time at this point. The local response itself is probably a depolarization of but a few ms. duration.

In the case of the ageing fiber or one responding with more highly damped oscillatory activity (B and C) the threshold is always much higher. This may be because the fiber is leaky (Wright, Coleman and Adelman, '55) and unable to maintain a proper potassium to sodium concentration ratio. It is not

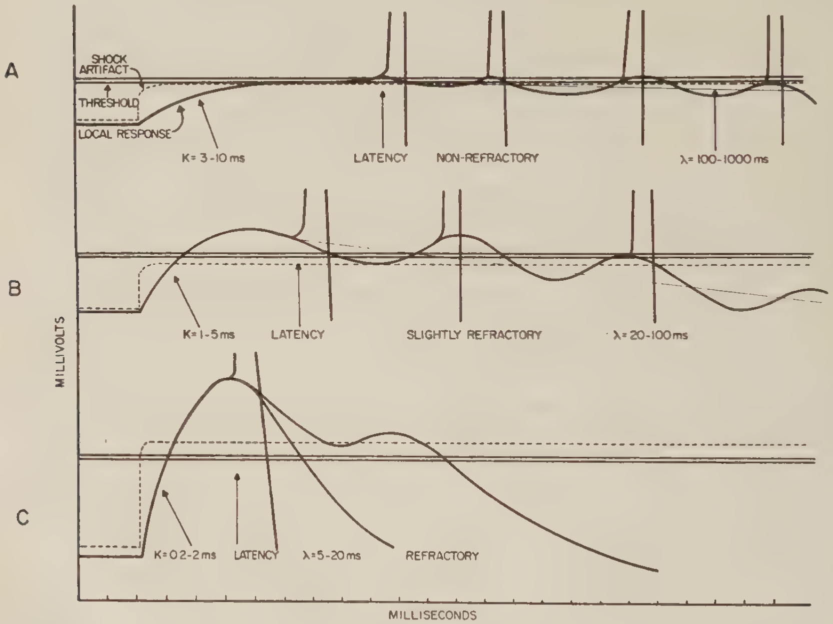

Fig. 11 Schematic diagram illustrating the trigger action of the local or subthreshold response elicited by DC stimulation in the highly excitable oscillatory responding fiber, (A), the moderately oscillatory responding fiber, (B) and the non-oscillatory responding fiber, (C). The shock artifact which normally obscures most of this response is purposely drawn in light dashed lines and the threshold is indicated by the double horizontal lines (see text).

unreasonable to suppose that the current required for stimulation allows sodium to enter the membrane and this large influx not only accounts for a local response of considerable magnitude, but tends to inactivate the sodium mechanism and cause the decay of the local potential. The fact that the fiber becomes refractory following such a response bears out this argument.

These subthreshold electrical events may be explained on the basis of the Hodgkin-Huxley axon model (Hodgkin and Huxley, '52) as shown in figure 12A. If a small resistance R_{Na_L} is included in series with the large resistance R_{Na} in the sodium circuit then with applied stimulation this smaller resistance is abruptly reduced first, allowing a small I_{Na_L} to flow and a small portion of the sodium potential E_{Na_L} (the local potential) to be recorded. When I_{Na_L} reaches some threshold value the larger R_{Na} is suddenly decreased and the total E_{Na_S} or the spike potential apears across the membrane. If it is assumed that $E_{Na_S} = E_{Na} - E_{Na_L}$, then obviously the greater the initial voltage E_{Na_L} the smaller the spike potential, E_{Na_S}, accounting for the decrease in spike height with increase in local response during fatigue. The sum of the spike and local response is less in the fatigued fiber than normally, (fig. 4), but this is probably because with continual stimulation and accumulation of Na ions in the fiber the total sodium potential, E_{Na} runs down and becomes smaller.

A physical picture of this electrical circuit may be visualized if one imagines two "layers of sites" within the membrane to which the Na ion may flow, an outer and an inner one (fig. 12B). An outer layer site must be totally exposed by the removal of several ions, perhaps by the withdrawal of a calcium ion (C) and a magnesium ion, (M), in order to make accessible the inner layer. These two ions are known to act synergistically (Adelman, '55). Partial exposure of the outer layer site may be accomplished with subthreshold stimulation removing only one ion and producing the collapse of the small sodium resistance R_{Na_L}. Subsequent Na ion movement to this opening gives the local response. To explain the oscillatory

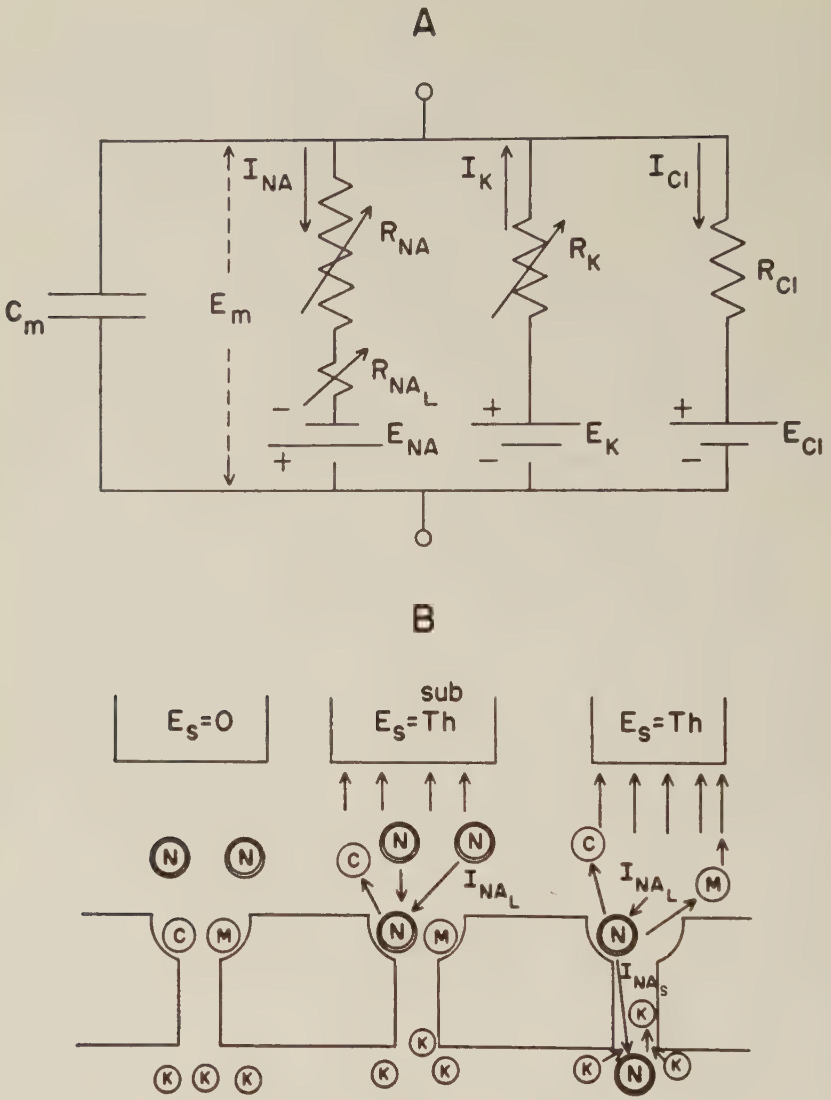

Fig. 12 A, Electrical circuit representing the fiber membrane, Hodgkin-Huxley model, with a small sodium resistance R_{Na_L} included in series with the larger R_{Na}. Collapse of R_{Na_L} allows a small sodium current to flow producing the local potential, E_{Na_L}. B. Schematic diagram of membrane at rest E = 0 (left), subjected to subthreshold stimulation, E = Subth (center) and threshold stimulation, E = th (right). An outer site is partially exposed by removal of one guardian ion (center) with subthreshold stimulation allowing small sodium current to flow. Path to inner sites is opened by threshold stimulus removing both guardian ions from the outer site. Then a large sodium current flows inwards through the membrane, and potassium starts outward.

nature of this response it must be assumed that the surface sites are exposed intermittently. At threshold a surge of Na current moves inward to other exposed sites producing the spike. Potassium (K) moves outward. Upon acquisition of all sites there is no other place for Na ions to flow, the current stops and the fiber is inactivated, refractory, until the sites are metabolically cleared of sodium.

SUMMARY

The subthreshold response to direct current stimulation of the crustacean (lobster) single motor axon has been studied with the following results:

1. The response may be different in voltage amplitude and duration, depending upon the type of fiber from which it is elicited, and the condition of the fiber.

a. The local response of the highly oscillatory responding fiber, the opener of the walking leg, is of a very low voltage, 1–2 mv., often not distinguishable from the shock artifact and of extremely long duration, over 15 msec.

b. The local response from the less oscillatory responding slow closer is of greater voltage, 2–5 mv., and shorter duration, 5–10 msec.

c. The local response of the non-oscillatory fiber, medial giant or fast closer axon, is of large voltage amplitude like a small spike, 15–20 msec. and of brief duration, 2–5 msec.

2. Deterioration or fatigue of a single axon transforms the local response from the low voltage long duration type to the high voltage short duration form.

3. Excess potassium in the medium surrounding the fiber increases the voltage and shortens the duration of the subthreshold response. The effect is similar to deterioration and fatigue.

4. Excitability curves obtained by the subthreshold dual shock method bear a striking resemblance to the contour of the local depolarization deflection. It is suggested that these curves actually are a graphical representation of the local depolarization.

5. The local response elicited by DC stimulation has a finite duration and turns off of its own accord. In the highly oscillatory fiber this turn-off is slow and is followed by little refractoriness, but in the non-oscillatory axons the turn-off is abrupt and subsequent fiber excitability is depressed by a significant refractoriness.

6. *Sodium in the surrounding medium is essential for the appearance of the subthreshold response. If sodium is absent all electrical activity in the fiber, local and all-or-none, is abolished.*

7. The local response is not affected by the cholinesterase inhibitor, eserine sulfate. A chemical mediator such as acetylcholine is probably not an essential step between applied electric currents and the local depolarization or trigger mechanism.

8. An explanation on the basis of the ionic theory of nerve activity for the various properties of the subthreshold response is presented.

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The author is greatly indebted to the very capable assistance of Dr. William J. Adelman, present address, Department of Physiology, University of Buffalo Medical School, Buffalo, New York, in the early portion of this work.

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THE HISTONES AND LIPOPROTEINS OF THE LIVER CELL NUCLEUS[1]

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THREE FIGURES

In the course of investigations concerned with the chemical nature of deoxyribonucleoproteins (Dounce and Monty, '55; Monty and Dounce, '58), we have become aware of several interesting properties of, and similarities between, the two major protein fractions (histone and lipoprotein) of rat liver cell nuclei isolated in aqueous media. The histone fraction and the lipoprotein fraction each can be subfractionated further into two classes of proteins differentiable by their solubility properties. The two histone fractions obtained from rat liver nuclei have solubility properties similar to those of the histone fractions obtained from calf thymus by Davison and Butler ('55) and by Daly and Mirsky ('55). The liver histone fractions, however, do not display the striking differences in amino acid composition reported for the thymus histones.

The present investigations deal in part with the preparation and properties of the liver histones. Amino acid and N-terminal amino acid analyses of the two fractions indicate their great similarity in composition. The properties of the two protein components of the non-histone portion of the deoxyribonucleoprotein (Monty and Dounce, '58) are also

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reported. Analyses reveal a close relationship in amino acid composition between these and the liver cell histones.

Isolation of cell nuclei

Three different methods of isolation were used for the purpose of comparison. Rat liver cell nuclei were isolated (a) at pH 6.0 in 1% gum arabic solutions (Dounce and Litt, '52), (b) at pH 6.0 in 0.44 molar sucrose solutions (Dounce, Witter, et al., '55) and (c) at pH 4.0 in dilute citric acid (Dounce, '55). In methods (a) and (c) the excised rat liver was stored overnight at —15°C prior to use. In method (b), liver from a freshly sacrificed animal was used.

Methods (b) and (c) produce nuclei in which the integrity of the deoxyribonucleoprotein has been largely preserved, as judged by their ability to form gels in dilute alkali (Dounce and Monty, '55; Monty and Dounce, '58). The nuclei prepared by method (a) have lost their gel-forming capacity.

Extraction of globulins

All operations were carried out at 0 to —5°C. An aliquot of nuclei representing a dry weight of 50 to 100 mg was centrifuged and resuspended in 25 ml of 0.9% NaCl. The pH of the suspension was immediately raised to 7.0 by the addition of dilute NaOH, using a glass electrode as indicator. The suspension was permitted to stand for half an hour with occasional agitation, and then centrifuged. The sediment was re-extracted in like manner for 15 minutes. The combined extracts contained the globulin fraction. Subsequent extractions did not yield further significant amounts of protein.

An alternative extraction procedure employed buffered saline solutions. In this case the extracting medium was 0.9% NaCl containing 0.05 molar phosphate buffer of pH 7.2. The extraction of globulins with this solution was performed as above, except that no pH adjustment was required.

The globulins in the saline extracts were coagulated by heating at 100°C for 15 minutes, and recovered by centrifugation. The coagulum was then washed twice with small volumes of distilled water, dried at 105°C, and weighed. Its weight was expressed as a per cent of the dry weight of the nuclei from which it was obtained.

Extraction of histones

The nuclear residue from which globulins had been extracted was washed twice with small volumes (approximately 10 ml) of distilled water to remove salts. The residue was then suspended in 15 ml of 0.2 N HCl and allowed to stand for half an hour. The mixture was centrifuged, and the insoluble portion reextracted as before for 15 minutes. The combined extracts contain the histones.

Fractionation of the histones

To the 0.2 N HCl extract was added one tenth volume of concentrated ammonium hydroxide. The solution was permitted to stand for half an hour, following which the ammonia-precipitable histone (P-histone) was collected by centrifugation. The addition of three volumes of 95% ethanol to the supernatant fluid from this centrifugation caused flocculation of the ammonia-soluble histone (S-histone). The precipitated S-histone was left in the cold for about 14 hours, and then was collected by centrifugation. Each histone fraction was dried at 105°C and weighed. Weights were expressed as percentages of the dry weight of nuclei from which the proteins were obtained.

The composition of liver cell nuclei with respect to proteins and deoxyribonucleic acid

Table 1 presents a summary of the data concerning the composition with respect to proteins and DNA of rat liver cell nuclei isolated by three different techniques. The residual material referred to is that nuclear material remaining after the extraction of globulins and histones, and contains protein,

TABLE 1

Analyses of isolated nuclei for proteins and DNA

GLOBULIN	NH_3-PPT'BLE HISTONE	SOLUBLE HISTONE	RATIO OF NH_3-PPT'BLE TO SOL. HISTONE	TOTAL HISTONE	RESIDUAL MATERIAL	DNA	RECOVERY
%	%	%		%	%	%	%
			Nuclei isolated at pH 6.0 in gum arabic				
20.0	22.3	5.2	4.3/1	27.5	35.0	11.8	82.0
13.9	18.5	5.3	3.4/1	23.8	26.9	—	64.6
13.3	25.3	5.9	4.3/1	31.2	22.2	—	66.7
10.1	25.6	6.7	3.8/1	32.3	41.4	12.9	83.8
9.8	26.6	6.6	4.0/1	33.2	40.1	13.4	83.1
13.4 (Ave.)	23.7	5.9	4.0/1	29.6	33.1	12.7	76.0
			Nuclei isolated at pH 6.0 in 0.44 M sucrose				
7.9	10.9	14.3[2]	0.76/1	25.2[1]	46.1	14.1	79.2
7.2	15.0	9.1[2]	1.65/1	24.1[1]	51.4	10.1	82.7
8.0	12.8	12.3[2]	1.04/1	25.1[1]	45.5	9.2	78.6
11.6	16.3	15.8[2]	1.03/1	32.1[1]	—	11.9	—
8.7 (Ave.)	13.7	12.9	1.12/1	26.6	47.7	11.3	80.1
			Nuclei isolated at pH 4.0 in dilute citric acid				
0[3]	19.7	3.9	5.0/1	23.6	66.0	13.3	92.0
7.6	19.9	6.5	3.1/1	26.4	68.0	12.4	102.0
5.3	19.3	6.0	3.2/1	25.3	62.8	13.2	93.4
11.2[4]	14.5	12.0	1.2/1	26.5	58.0	12.2	95.7
11.0[4]	15.2	10.7	1.4/1	25.7	58.6	—	95.5
12.6[4]	14.2	13.8	1.0/1	28.0	51.2	13.5	91.8
13.1[4]	13.9	11.4	1.2/1	25.3	50.6	11.9	89.0
Ave.	16.5	9.5	2.3/1	26.1	62.2	12.7	94.2

[1] Determined by Kjeldahl analysis of the HCl extract.

[2] Determined by the difference between total histone and NH_3-precipitable histone.

[3] No pH adjustment was made during the saline extraction.

[4] Globulins were extracted with the buffered saline solution.

lipid, and nucleic acid. DNA was determined on aliquots of whole nuclei by the method of Schneider ('45) using the diphenylamine reaction.

A fairly constant ratio of histone to DNA, with a numerical value of about two, was obtained in all experiments. The amount of residual material was higher in the nuclei isolated at pH 4.0 than in the other preparations, perhaps reflecting some loss in solubility of globulins caused by partial denaturation by the low pH of the isolation medium. In this connection it is of interest to note the inverse relationship between globulins and residual material in these nuclei isolated at pH 4.0.

The most striking variation in these experiments is in the relative amounts of the two histone fractions. While the total amount of histone remained constant, the ratio between the two histone fractions varied with the conditions of nuclear isolation and with the method of globulin extraction.

Properties of the liver histones

The two histone fractions were obtained in quantity from nuclei isolated at pH 4.0, from which the globulins had been removed by extraction with buffered saline. The P-histone was only partially soluble in distilled water, but dissolved with ease in 0.1 N HCl. The S-histone was found to be rapidly and completely soluble in both distilled water and dilute acid. Lyophilization did not appear to have a detrimental effect on the solubility properties of either histone fraction. Both histones were soluble in both hot and cold solution of 0.17 M $HgSO_4$ in 0.94 M H_2SO_4. This solubility behavior has been described by Mirsky and Pollister ('46) as specific for the histones.

Small samples of the two histone fractions were hydrolyzed in 6 N HCl for 14 hours in sealed tubes, and the neutralized hydrolysates were subjected to two-dimensional paper chromatographic analysis. The solvent systems employed were phenol saturated with water in the presence of ammonia, followed by collidine lutidine (50/50) saturated with water and run in the presence of diethylamine. The chromatograms were developed by spraying with ninhydrin.

Identified by their positions on the chromatogram and the color produced by their reaction with ninhydrin were aspartic acid, glutamic acid, glycine, serine, threonine, alanine, lysine, arginine, histidine, proline, valine, leucines, phenylalanine, and tyrosine. The presence of cysteine and methionine was demonstrated in chromatograms of peroxide-treated hydrolysates. In addition, an amino acid tentatively identified as γ-aminobutyric acid was observed. This amino acid behaved in a fashion apparently identical with synthetic γ-aminobutyric acid with respect to both chromatographic characteristics and reaction with ninhydrin. No further attempts were made to confirm the identity of this material.

The amino acid composition of the two histones is essentially identical, as can be seen by a comparison of the chromatograms of figures 1 and 2. This similarity extended to the inclusion in both proteins of the amino acid identified as γ-aminobutyric acid.

Samples of the histone fraction were then reacted with 2,4-dinitrofluorobenzene by the method of Sanger ('45). The dinitrophenyl histones were recovered by filtration, and washed with distilled water, ethanol, and ether respectively until no further color was extracted by any of the solvents. The histone derivatives were then hydrolyzed with 6 N HCl for 8 hours at 105°C in sealed tubes. That a quantitative reaction had taken place between lysine, histidine and tyrosine and the dinitroflurobenzene was confirmed by two-dimensional paper chromatography, performed as described above. No other amino acids were missing or even markedly depleted in the ninhydrin-treated chromatogram as indicated by comparison with chromatograms of the hydrolysates of the original untreated histone fractions. The amino acid tentatively identified as γ-aminobutyric acid appeared as a free compound in the hydrolysates of the dinitrophenyl histones, implying that its amino group was not free within the protein molecules.

To separate the dinitrophenyl derivatives, a uni-dimensional chromatogram developed with dioxane/ammonia (4/1) on acid-washed Whatman no. 1 filter paper was made. This indicated the presence of at least 5 different DNP-amino

Fig. 1 Paper chromatogram of hydrolysate of ammonia-precipitable histone fraction. Phenol-water, right to left, in horizontal direction, with collidine-lutidine-water in vertical direction. Chromatogram developed with ninhydrin.

acids in the hydrolysates of each dinitrophenyl-histone. An ether extract of the acidified hydrolysate contained at least three, and possibly 4, different DNP-amino acids. Apparently identical results were obtained with the two histone fractions. Identification of the DNP-amino acids was not undertaken.

Fig. 2 Paper chromatogram of hydrolysate of ammonia-soluble, alcohol-precipitable histone. Conditions same as for figure 1.

Studies of histone aggregation

The two histone fractions thus seem to have similar amino acid compositions, and yield the same products on reaction with dinitrofluorobenzene. It seemed likely, therefore, that the two histones might represent merely different states of aggregation of the same proteins.

The variable ratio of the histones in isolated nuclei suggested that some sort of interconversion of the histones could occur. To examine this possibility, the following experiments were performed. Histones were prepared in the usual fashion from nuclei isolated at pH 4.0. Each of the two fractions was redissolved in 0.2 N HCl and reprecipitated by the appropriate means. The samples were then dissolved in 0.2 N HCl, and dialyzed against distilled water overnight.

Samples of each dialyzed histone fraction were incubated at 0°C for one hour in either distilled water or 0.9% NaCl buffered with 0.05 M phosphate buffer of pH 7.1. At the end of the incubation, the P-histone and S-histone in each sample were recovered by precipitation, dried, and weighed. The results appear in table 2 A.

A second experiment involved incubation of the histone fractions in 0.9% saline adjusted to pH 7.0 with NaOH, 0.9% NaCl containing 0.05 M phosphate buffer of pH 7.1, or 1 M NaCl adjusted to pH 7.0 with NaOH. The incubation was for 12 hours at 0°C. The results are presented in table 2 B.

During the incubation of P-histone in salt solution, considerable flocculation occurred. No flocculation was observed in the solutions of S-histone under any of the conditions employed.

From a consideration of table 2, it would appear that some conversion of P-histone to S-histone occurs in salt solutions at neutrality, but it is doubtful if the reverse occurs. The higher salt concentration seems most favorable to the conversion. The presence of phosphate during the incubation had no apparent effect. Concomitant with the release of S-histone from P-histone under these conditions is an apparent formation of higher states of aggregation of some of the P-histone, as evidenced by the flocculation which occured in these solutions.

In contrast to these results, a mutual interconversion of the two histones appears to occur in water. The pH of the incubations in water was not determined accurately because of the low buffering strength, but was below neutrality.

TABLE 2

A. Interconvertibility of the histone fractions in water and in physiological saline at pH 7.0

HISTONE FRACTION USED AS STARTING MATERIAL	INCUBATION MEDIUM	WT. RECOVERED BY NH_3 PPT'N.	WT. RECOVERED BY ALCOHOL PPT'N	TOTAL WT. RECOVERED
		mg	*mg*	*mg*
NH_3-ppt'ble histone	Buffered saline	11.9	2.9	14.8
	Water	8.6	3.7	12.3
Soluble histone	Buffered saline	0.7	8.3	9.0
	Water	1.2	5.8	7.0

B. Interconvertibility of the histone fractions in salt solutions at pH 7.0

NH_3-ppt'ble histone	0.9% saline	12.2	2.6	14.8
	Buffered saline	12.4	3.0	15.4
	1 M NaCl	11.6	4.0	15.6
Soluble histone	0.9% saline	0	3.0	3.0
	Buffered saline	0	3.3	3.3
	1 M NaCl	0	4.0	4.0

The nuclear lipoproteins

We have demonstrated earlier (Monty and Dounce, '58) that the lipoprotein normally bound to DNA in the liver cell nucleus can be separated into two fractions by heating. The majority of the lipoprotein remains relatively insoluble, but a discrete fraction, referred to as the "occult protein," is solubilized. This "occult protein" has solubility properties similar to those of S-histone, remaining in solution over the range of pH 1 to pH 10. It is precipitable from 10% NH_4OH by the addition of ethanol. It differs from the "native" histones in being insoluble at all temperatures in the mercuric sulfate-sulfuric acid reagent (Mirsky and Pollister, '46), but it might represent a firmly found histone which is denatured during the process of being extracted with hot HCl.

An examination by paper chromatography of the amino acids of the total lipoprotein fraction and of its two compo-

Fig. 3 Paper chromatogram of hydrolysate of the residual deoxyribonucleolipoprotein fraction, using the same conditions as for figures 1 and 2. The ''occult'' protein fraction has not been extracted.

nents failed to reveal any significant differences between the three materials. The chromatogram obtained from an acid hydrolysate of total lipoprotein is reproduced in figure 3. These proteins were also observed to contain the amino acid demonstrated in the histones and tentatively identified as γ-aminobutyric acid.

It was surprising to note the remarkable similarity between the histones and the lipoproteins in their amino acid compositions. The only significant difference observable was the possibility of a slightly higher content of arginine and lysine in the histones. The globulins of rat liver nuclei, by contrast, display a preponderance of neutral amino acids, and chromatograms of their hydrolysates are easily distinguished from those of the histones and lipoprotein.

DISCUSSION

The two histones from isolated rat liver cell nuclei are found to display very similar compositions with respect to amino acids. In addition, the N-terminal residues appear to be the same. The apparent interconvertibility of the histones, then, may be due to changes in the state of aggregation rather than to autolytic or proteolytic phenomena.

It is interesting to compare the two histones from liver cell nuclei with the histone fractions prepared from calf thymus nuclei by Davison and Butler ('55) and by Daly and Mirsky ('55). The calf-thymus histones are also separable by precipitation with ammonia, although here higher concentrations of ammonia are required. In contrast with those of liver, the two thymus histones display profound differences in amino acid composition (Davison and Butler, '55; Daly and Mirsky, '55). Phillips ('58) has demonstrated that they also have different N-terminal amino acid residues.

The present data indicate that a wide divergence in amino acid composition does not occur in the two histone fractions from rat liver. While the analytical methods employed in this investigation are admittedly only semi-quantitative, chemical differences such as have been reported for the thymus histones could be obscured only by much more extensive cross-contamination than seems plausible. Why greater differences should occur between the P and S histones of thymus than between the P and S histones of rat liver cannot be stated, but it is possible that more autolytic proteolysis might occur in the case of thymus than in the case of liver.

In regard to possible interconversion of the P and S histones, it was found that incubation in neutral salt solutions resulted in the release of some S-histone but the reverse apparently did not occur. However, some evidence for the conversion of S-histone to P-histone was obtained after incubation of the former in water at a pH below 7.

Cruft et al. ('54) obtained electrophoretic evidence for the aggregation of histones, and the histones of thymus have been shown by Davison et al. ('54) to form aggregates under a variety of conditions. In this latter case however, it is the ammonia-precipitable histone which appears to be primarily responsible for the aggregation phenomenon, and therefore a higher order of aggregation than the conversion of S-histone to P-histone must be under study.

It is likely that something present in nuclear material can influence the ratio of P histone to S histone, since in interconversion experiments starting from P- or S-histone, it was not possible to produce the same ratios of the histone fractions as were obtained by extraction of isolated nuclei. In this connection, it is also of interest that a ratio of unity for P and S histone was obtained only with nuclei in which the nucleoprotein had been protected from damage by mitochondrial factors previously shown to induce the cleavage of DNA from lipoprotein. In what manner such cleavage might affect the ratio of P and S histone cannot however be stated, nor can it be said whether mitochondrial factors might be able to affect the ratio. The nuclear lipoproteins have relatively low isoelectric points between pH 5 and 6 for liver cell nuclei (Wang et al., '53), and at a somewhat lower pH for chicken erythrocyte nuclei (Engbring and Laskowski, '53) In spite of this, amino acid chromatograms of the histones and the lipoproteins were found to be essentially indistinguishable from one another. Hamer ('53) has pointed out a similarity in composition of the histones and the non-histone residual proteins obtained from thymus nuclei, and has emphasized that the primary differences were the lower levels of cystine and tryptophane in the histones. One is led to suspect therefore that the acidic nature of the lipoproteins might be dependent to a considerable extent upon the lipid moieties. Relatively little information is available concerning the nature of these lipids, except that they constitute about 10% of the lipoprotein molecule and include both cholesterol and phospholipid (Wang et al., '53). On the other hand, it is also possible that the histones may contain more amide groups than the residual protein.

It has been suggested that the DNA-lipoprotein may serve as basic structural material in the chromatin threads (Monty and Dounce, '58). The histones apparently associate with this nucleoprotein macro-molecule chiefly by ionic bonding. However, no function has been securely established for the histones, in spite of their widespread occurrence in cell nuclei, although Cruft et al. ('54) have suggested that they might act as gene inhibitors. We should like to suggest another possibility, namely that the histones, in addition to neutralizing charges in DNA molecules, might serve as basic units which polymerize at some time during the mitotic cycle to form the protein component of the lipoprotein (residual protein) of the nuclei. This suggestion is not contradicted by the fact that histone apparently is synthesized concomitantly with DNA (Bloch and Godman, '55), and it seems to fit in with the result reported in this paper. However there are a number of different observations which on the surface seem not to be in agreement with such a concept, and hence the idea can be advanced only very tentatively.

SUMMARY

1. Two histone fractions have been obtained from isolated rat liver cell nuclei, and have been compared in amino acid composition with the residual lipoprotein and a component of the latter termed "the occult protein."

2. The solubility properties of the two histone fractions resemble the solubility properties of corresponding histone fractions obtained from calf thymus, but the amino acid composition of the two liver histones are similar or identical, whereas the two thymus histones have been shown to differ widely in amino acid composition. Analysis with dinitrofluorobenzene indicates that the same N-terminal amino acids are present in both of the liver histone fractions.

3. The amino acid compositions of the two rat liver histones are very similar to the amino acid composition of the residual lipoprotein and of the "occult" protein.

4. The ratio between the two histone fractions has been shown to vary, depending on the method of isolation of the nuclei, but the sum of the two fractions remains constant.

5. In salt solution some conversion of the more insoluble liver histone to the more soluble liver histone can be shown to occur *in vitro* but the reverse of this does not occur in salt solution. A certain degree of mutual interconversion of the two histones appears to occur in distilled water solution, however.

6. It is suggested that the two histones of rat liver nuclei may differ chiefly in their states of molecular aggregation and that histone molecules might possibly serve as structural units which through polymerization bring about synthesis of the protein component of the lipoprotein of the nuclei, (which is also termed the "residual protein").

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UREA EXCRETION AND DIVING IN THE SEAL (PHOCA VITULINA L.)[1]

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FIVE FIGURES

The seal shows interesting physiological adaptations to its environment and diving habits. To the renal physiologist the circulatory changes that take place during diving and the changes in renal function accompanying feeding have been of particular interest.

It was shown by Hiatt and Hiatt ('42) that the glomerular filtration rate (GFR) and renal plasma flow (RPF) increase considerably three to 4 hours after feeding. The lability of the renal function of the seal was further demonstrated by Bradley and Bing ('42) who found that the GFR and RPF could be reduced to approximately one fifth of the resting value if the respiration was arrested (simulated diving) by holding a cone over the nostrils of the seal.

Findings on the urea excretion of the seal by Smith ('36) suggested to us that the urea clearance might also be more labile than in other mammals. From Smith's data it can be calculated that in the fasting seal the urea/inulin clearance ratio is quite low (5–8%). Furthermore, Smith ('36) found that the ingestion of 1 kg of herring caused the urine flow to increase 10-fold while the urea concentration in the urine simultaneously increased approximately 5-fold. This means a 50-fold increase in the rate of urea excretion. Combining this observation with the observation by Hiatt and Hiatt that the glomerular filtration rate approximately doubles after the ingestion of 1 kg of herring, we can estimate that the concentration index (inulin U/P ratio) of the urine probably decreased to ⅕ of its value prior to feeding. This should cause the urine urea concentration to decrease, but instead a 5-fold increase was found. This could of course be caused by an increase in the plasma urea concentration (25-fold), but it would also be quite possible that the urea clearance increased out of proportion to the increase found in the glomerular filtration rate.

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In many mammals urea excretion has been found to be regulated on the tubular level (Schmidt-Nielsen, '58). When the animals are placed on a low nitrogen intake for a period of time the urea clearance decreases relative to the filtration rate and the maximum urea U/P ratio that can be attained becomes markedly lowered. This type of regulation is particularly pronounced in ruminants (Schmidt-Nielsen, Schmidt-Nielsen, Houpt, and Jarnum, '57; Schmidt-Nielsen and Osaki, '58; Schmidt-Nielsen, Osaki, Murdaugh and O'Dell, '58) where the maximum urea U/P ratio during normal nitrogen intake is approximately 300 while during low nitrogen intake it may not exceed 5–7. Therefore, there were reasons to suspect that in the seal the urea excretion might be regulated in a similar manner but with changes occurring with each feeding.

The results of the present investigation show that the urea/inulin clearance ratio in the seal does increase after feeding. The increase, however, is dependent upon an increase in urine flow and is due to the marked variation of the urea clearance with urine flow. Thus, the variation is of a different nature from the regulated changes found in the other mammals during prolonged alterations in nitrogen intake.

It has been demonstrated by Irving et al. ('36), Scholander ('40), Irving and Martin ('42) and Grinnell et al. ('42) that pronounced bradycardia develops in the seal when breathing is arrested and that under such conditions the peripheral blood flow is reduced to a minimum. The mechanism serves to conserve oxygen for the vital organs, and blood flow to the brain is increased in apnea.

The finding that the RPF and GFR were reduced during simulated diving indicated that the blood flow through the kidney during diving is reduced similarly to the blood flow through the muscles. One factor, however, that has not been taken fully into account is that the physiological effects of simulated diving accompanied by fighting and struggle is not identical to the effects of normal diving.

Since the renal function supposedly is reduced to a minimum during diving it is of interest to know how much time the seal normally spends under water in 24 hours. Therefore, the general behavior, i.e., feeding and diving pattern, of the captive seals, was recorded in the present study. Diving experiments were carried out in order to study the effect of different types of diving upon the heart and renal function. In a 24-hour period the seal was found to spend more than one third of its time under water. The effect on the heart was found to differ in quiet respiratory arrest from that found during forced apnea accompanied by struggle. The effects on the renal hemodynamics cannot be conclusively ascertained from our limited data. It is evident, however, that excitement and struggle in itself, whether accompanied by apnea or not, affect the renal function and particularly the urea excretion.

METHODS

Six young female harbor seals (*Phoca vitulina* L.) were used. They had been caught in the open sea shortly after weaning. Body weights were between 20 and 22 kg. During the months of July and August when the experiments were performed they were kept in open live cars partly immersed in sea water. They were fed fresh herring which they took readily from the hand. Their appetites were good and each seal consumed on the average 2½ kg of herring per day. Since seals are liable to serious infections and septicemia in captivity, Bicillin (600,000 units) was administered to each seal every three weeks.

In the majority of the experiments the GFR was measured as the inulin clearance, and the RPF as the para-aminohippurate (PAH) clearance. The seals were taken into the laboratory and placed in a canvas sling in the prone position. In this position, which resembles the normal position in water, the animals were usually quiet and cooperative. Singing to the animals was found to be effective in distracting their attention from the experimental procedures. Inulin (20 mg/min.) and PAH (3.5 mg/min.) in normal saline were infused continuously through a polyethelene catheter inserted in a vein in a front flipper. The animal was catheterized with a Foley catheter (size 12). Blood samples were collected from veins in the hind flippers approximately every 60 minutes. Urine samples were collected from the indwelling catheter every 30–60 minutes. Throughout an experiment, which usually lasted 5–8 hours, every effort was made to make the procedure as nontraumatic to the seal as possible.

Simulated diving experiments were performed in the laboratory during infusion by using two different methods. The first method was to trickle water over the nose of the seal. This caused the seal to close the nostrils voluntarily for periods up to two to three minutes. The seals struggled much less when apnea was induced in this manner. Sometimes they were completely quiet, but seldom long enough to provide time for a good renal clearance determination. Respiration was also arrested in a manner similar to that used by other investigators in which a closed funnel was held firmly over the nose of the seal. This form of apnea resulted in violent fighting on the part of the seal.

True diving experiments were performed in the live car. In these experiments the endogenous or exogenous creatinine clearance (creatinine administered by stomach tube) was used as a measure of the GFR. An indwelling catheter was placed in the bladder but no infusion was given. The animal was left in the live car, free and unhampered. Every 30–60 minutes it was lifted up on the dock and urine and blood samples were collected as fast as possible. During the time in the live car the animal was kept under constant observation and the time spent under and above water was continuously recorded.

The exogenous creatinine clearance appears to be a rather reliable measure for the GFR in the seal. In experiments by Smith ('36) where both creatinine and inulin were given subcutaneously the creatinine/inulin clearance ratio was found to be 0.98 (average of 5 clearance periods). In those of our experiments where inulin was administered by continuous infusion and creatinine was given by stomach tube, the average of 9 clearance periods gave a creatinine/inulin clearance ratio of 1.02 ± 0.12 (S.D.). The endogenous true creatinine (ETC) clearance which was compared with the inulin clearance in 28 periods was found to be consistently lower than the inulin clearance. The average ETC/inulin clearance ratio being 0.75 ± 0.07 (S. D.).

Analytical methods

Urea and ammonia were determined according to Conway's microdiffusion method ('42); endogenous true creatinine according to Hare's method ('50) and exogenous creatinine according to the same method but without Lloyd's reagent; inulin by the resorcinol method according to West and Rapoport ('49); para-aminohippurate (PAH) by the method of Selkurt ('48); sodium and potassium with a Baird Flamephotometer.

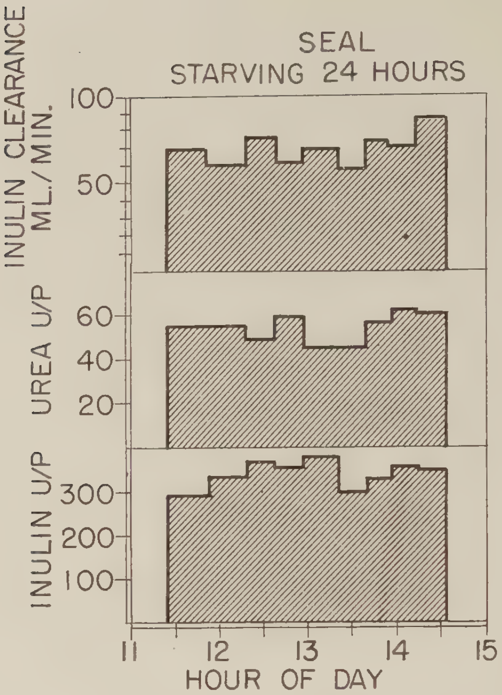

Fig. 1 Starved seal. Constant infusion in laboratory. Infusion started at 0947. No treatment during experiment. Plasma urea concentration remained around 13 mM/liter throughout the 5 hours of observation.

RESULTS

Fasting and feeding

In a seal fasted for 24–36 hours the GFR was found to average 66 ml per min. (fig. 1 and table 1). Three to 4 hours following the ingestion of 1 kg of herring the GFR increased approximately 100% (fig. 2 and table 1), the magnitude of the increase being the same as that observed by Hiatt and Hiatt ('42).

The plasma urea concentration increased from values of about 12 mM/liter in the fasting seal to 17 mM/liter 3–4 hours after feeding. The plasma sodium and potassium concentrations did not change appreciably.

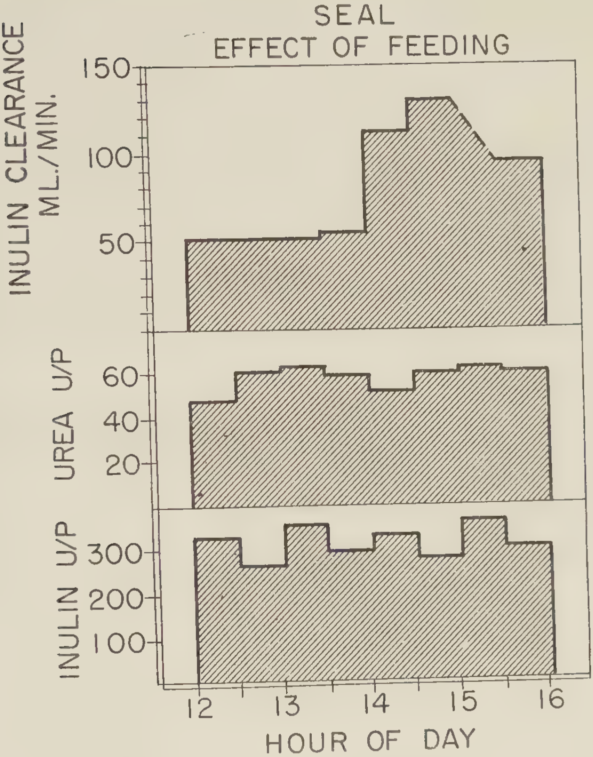

Fig. 2 The effect of feeding in the seal. The seal was fed at 10 o'clock. The infusion started at 1117. The plasma urea concentration rose continuously from 15.4 mM/l at 1050 to 18.85 mM/l at 1610 o'clock. The filtration fraction (inulin/PAH clearance ratio) averaged 0.430 and did not change throughout the experiment. It is seen that the urea U/P ratio remains unchanged in spite of the marked increase in filtration rate.

The urine concentrations of urea, sodium and potassium increased after feeding. In the present experiments the concentration index (inulin or creatinine U/P ratio) was practically the same in the fed and fasting seal. This, however, does not mean that the same is the case in the undisturbed seal. The spontaneous rate of urine flow was normally considerably lower in the fasting than in the fed seal. The infusion and the handling of the seal caused some changes in the urine flow and solute excretion.

TABLE 1

EXP. NO.	URINE CONC.			PLASMA CONC.			GFR	FF	INULIN OR CREATININE	UREA
	Urea	Na+	K+	Urea	Na+	K+				
	mMol/l	*mEq/l*	*mEq/l*				*ml/min.*	$\frac{inulin\ cl.}{PAH\ cl.}$	*U/P*	*U/P*
				After 24 hours of fasting						
1	735	44	32	12.2	144	3.5				10.2
	672	80	60	12.2	143	3.5				55.1
	654	—	80	11.5	142	3.5				56.9
	666	106	96	11.5	142	3.5				57.9
2	605	188	33	11.1	152	3.2	70.6		290	54.2
	706	125	46	12.9	152	3.2	60.8		331	54.7
	666	88	36	13.6	152	3.2	76.2		362	48.9
	822	76	46	13.8	152	3.2	67.0		355	59.6
	638	109	49	14.0	152	3.2	68.6		373	45.6
	634	100	31	14.1	152	3.2	57.3		295	45.0
	784	84	50	13.9	152	3.2	73.2		325	56.4
	870	100	44	13.9	152	3.2	70.3		351	62.6
	845	138	32	13.8	152	3.2	88.7		347	61.2
3	559	77	—	13.2	147	5.2	72.0		368	42.3
	677	50	75	12.5	147	5.2			256	54.2
4	681	16	19	13.0	150	3.3	67.0		343	52.4
	878	11	7.5	13.4	150	3.3	62.5		372	65.5
5	533	18	19	9.58	157	3.1	65.4		327	55.6
	375	27	15	9.46	156	3.1	48.3		372	39.6
	419	25	13	9.27	156	3.1	51.5		431	45.2
Av.	671	77	41	12.5	150	3.5	66.3		343	53.7
				4–8 hours after the ingestion of 1 kg of herring						
6	913.5	107.6	134	17.6	156	3.9	113.5	0.363	330	51.9
	1077	154.0	164	17.8	156	—	129.4	0.394	272	60.5
	1134	—	117	18.1	156	—	—	0.445	365	62.6
	1132	—	128	18.5	156	—	94.9	0.364	302	61.2
7	1188	150	75	17.0	138	3.8	121.1	0.547	223.5	69.8
	1068	99	149	17.8	135	3.8	103.4	0.479	200.0	60.0
8	672.91	121	53.5	13.6	157	3.1	—	—	756.0	49.4
Av.	1026	126	117	17.2	151	3.7	112.5	0.432	350	52.3

	Saline infusion; 5.33 ml/min. of 0.9% saline for 3 hours								
9	221.9			10.0	158	3.1				22.2
	402		82	10.4	158	3.3				38.7
	246	250	112	10.9	158	3.3				22.6
	333	1465	102	11.2	158	3.3	57.2	0.278	231	29.7
	441	326	33	11.2	158	3.4	66.9	0.309	162	39.4
	397	298	22	10.7	158	3.6	72.8	—	122	37.1
	437	405	24	10.3	158	3.7	85.8	0.306	98.8	42.4
	215	286	16	9.73	158	3.8	87.7	0.310	44.2	22.1
	171	240	17	9.18	158	3.8	84.5	0.310	33.4	18.6
	162.1	191.5	15	8.58	158	3.8	87.8	0.258	38.3	18.9
	153.2	149.5	14	7.90	158	3.8	79.2	0.350	33.3	19.4
	151.0	121.5	15	7.78	158	3.8	86.5	0.355	37.4	19.4
	123.0	50.8	16	7.67	158	3.8	71.7	0.205	34.1	16.0
	187.8	86.7	26	7.25	159	3.8	74.2	0.298	62.3	25.9
	223.5	173.5	32	6.85	159	3.8	—	0.302	102.0	32.6
Av.	257.6	209.5	37.6	9.31	159	3.6	77.7	0.298	83.2	26.9
	Urea infusion; 3.22 ml/min. 4% urea for 4 hours									
10	568	17.2	10.3	20.0	164	—	36.1		78.5	28.4
	163.5	8.8	2.1	31.0	160	—	—	0.338	11.2	5.27
	139.8	9.0	1.7	35.0	154	—	60.6	0.287	6.57	3.99
	153.4	19.0	2.0	39.0	155	—	67.6	0.272	6.57	3.93
	288.8	40.1	3.6	40.0	156	—	63.9	0.315	12.68	5.72
	330	66.8	6.5	37.0	155	—	74.8	0.325	18.90	8.92
	357	78.1	—	32.7	161	—	68.7	—	20.05	10.9
Av.	277.2	34.1	4.4	33.5	157		62.0	0.307	22.06	9.59
	"Water" infusion; 3.22 ml/min. of 5% dextrose for 2½ hours									
11	820	12.2	26.4	13.2			58.0		205	
	651	—	13	13.6					169.5	47.9
	424	—	—	13.3			51.4		113.8	31.9
	314	—	—	13.1						24.0
	250.3	—	—	13.0						19.2
	215.6	—	2.3	14.4			67.0		75.0	15.0
	206.0	3.8	2.3	13.9			64.0		74.7	14.8
	277.8	4.0	2.4	13.3			58.0		81.0	20.9
	270.0	4.3	5.1	12.9			56.6		71.4	20.9
Av.	381	6.1	8.6	13.4			58.3		112.9	24.3

At low urine flows the urea U/P ratio was found to be remarkably constant around 50 to 60. From figures 3 and 4 it can be seen that when the urea/inulin clearance ratio or the urea U/P ratio is plotted against the concentration index the data obtained in the fed and fasted seal overlap completely. In other words, in spite of differences in filtration rate and plasma urea concentration the fraction of filtered urea excreted does not vary with feeding but varies only with the inulin U/P ratio.

Urea, saline, and water infusion

Experiments were carried out in order to find if the effect of feeding could be due to the intake of salt or water contained in the herring, or to the urea formed in its metabolism.

From table 1 it can be seen that neither of the three substances caused an increase in the GFR similar to that caused by feeding, but the urine flow increased considerably in all three experiments.[5]

The data obtained on the urea excretion are plotted together with the previously discussed data in figures 3 and 4. It is seen that the data fall on the same general curve. The urea/inulin clearance ratio is highly dependent upon the tubular reabsorption of water (as represented by the inulin U/P ratio) and consequently the urea excretion varies markedly with urine flow. This is due to the fact that a maximum urea U/P ratio (50–60) already is reached at an inulin U/P ratio of about 150–200 (fig. 4).

From figures 1, 2 and 4 it can be seen that the maximum urea U/P is of the same magnitude in the fasting and in the fed seal. Because of the constancy of the urea U/P ratio at low urine flows (inulin U/P > 150), it is more convenient to use the maximum urea U/P ratio rather than the urea/inulin clearance ratio as an indicator of the renal handling of urea.

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[5] It is curious that infusion of urea in saline caused marked hemolysis in the seal, even when the increase in plasma urea concentration was moderate (30 mM/l). In the experiments by Page et al. ('54) death occurred as a result of massive hemolysis after urea in saline was infused. According to our observations in sheep, hemolysis will occur after infusion causing only a 5–10 mM/l increase in the plasma urea concentration. Hemolysis does not occur *in vitro* when red cells from these animals are mixed with urea saline solutions. Hemolysis does not occur in dogs after infusion of urea in saline.

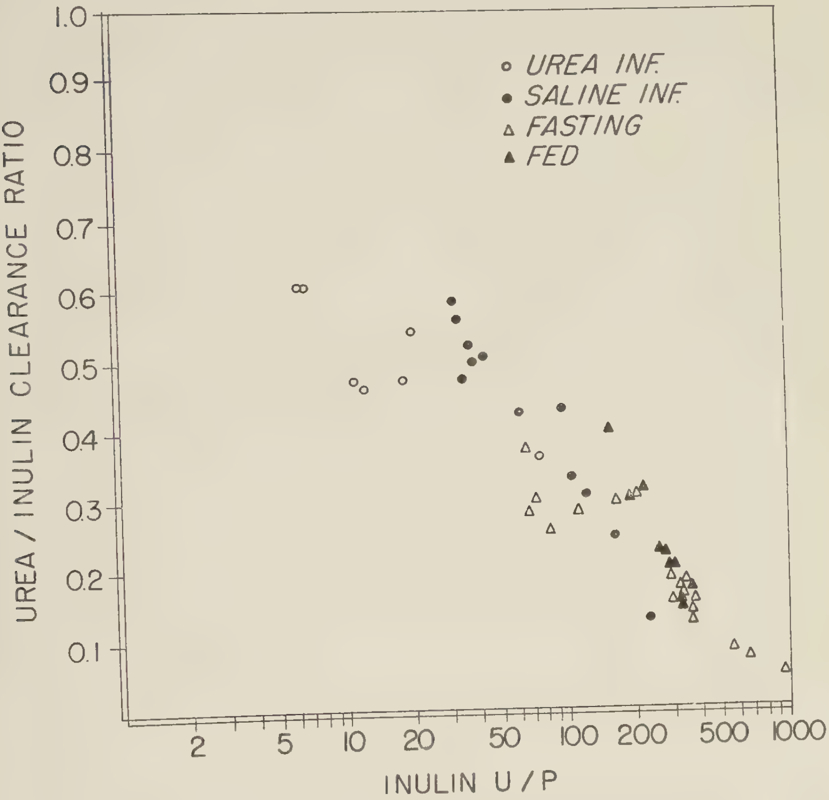

Fig. 3 The urea/inulin clearance ratio, representing the fraction of filtered urea which appears in the urine, plotted against the inulin U/P ratio. The plasma urea concentration, glomerular filtration rate and filtration fractions for the plotted data are given in table 1.

General behavior of the seal; feeding and diving pattern in captivity

The seals were fed twice a day, in the morning at 8 A.M. and in the evening at 8 P.M. After feeding, the seals would be relatively lively, swimming and diving in the live car for about an hour. In this period the seals would be under water for short periods of up to 50 seconds. They would have the head above the water for periods varying from 5 to 60 seconds. If undisturbed the seals normally went to sleep during the second hour after feeding. During sleep the seals rested immobile on the bottom of the cage under water with the eyes closed. The average period of time spent under water during the deepest sleep was 2½ to 3 minutes. The maximum single period that we observed a seal to stay under water was 4 minutes and 13 seconds. During sleep the seal would slowly move to the surface and, with its eyes still closed, respire approximately 15 times before it would again sink back to the bottom of the

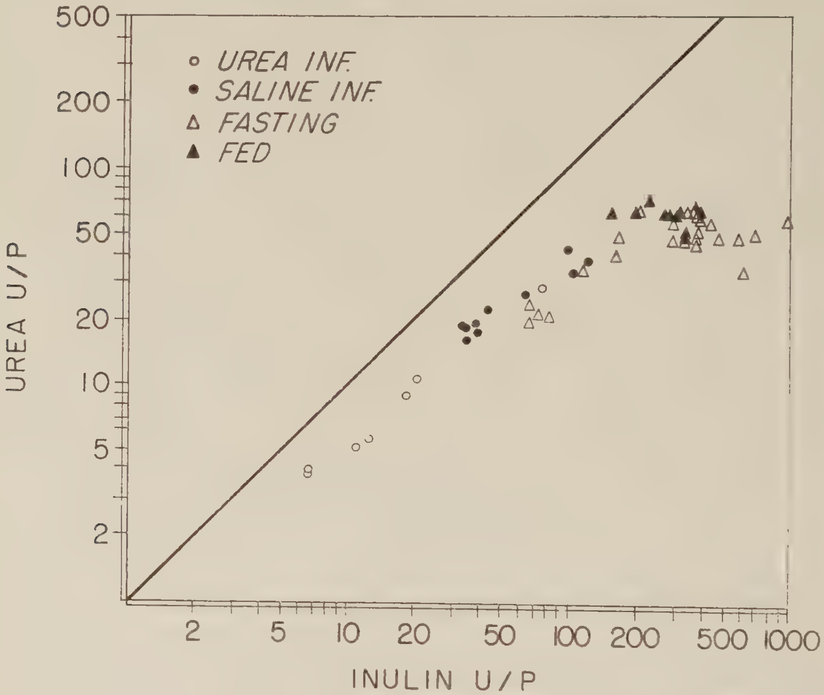

Fig. 4 The same data as in figure 3 plotted as urea U/P against inulin U/P ratio. The urea U/P ratio becomes constant at an inulin U/P ratio of approximately 150. The data indicate a poor ability of the seal kidney to concentrate urea in the urine.

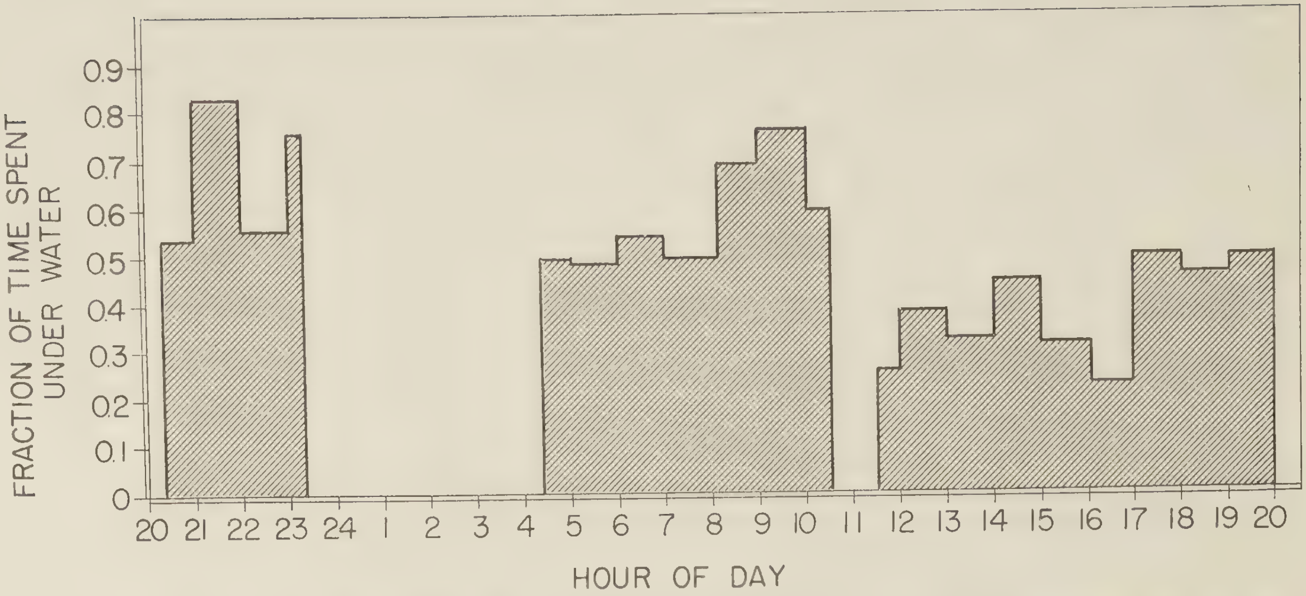

Fig. 5 Diving pattern in seal observed through 24 hours. The fraction of time spent under water is given. The seal was fed at 2000 and at 800 o'clock. The length of each individual dive varied from a few seconds up to 4 minutes.

cage. After one to two hours of sleep the seals usually became lively and started to swim around playfully in the cage.

The recorded observations on the diving pattern of one seal are presented in figure 5. In a seal that was observed continuously during a 24 hour period the total amount of time spent under water was 8 hours 30 minutes, or 35.5% of the time. The maximum amount of time spent under water in a one hour period was 83% in one seal and 88% in another.

This diving pattern observed in captivity may not be identical to the pattern under natural conditions. Since captive seals do not hunt their food under water there is no incentive to remain submerged for long periods of time. The maximum length of time a seal can safely spend under water was found by Scholander ('40) to be approximately 20 minutes. Such long periods of diving were never observed in our captive seals. When the seals were swimming fast the longest diving periods were of the order of one minute, and only when the seals were sleeping did we observe diving periods of three to 4 minutes. However, even though the seal in the open sea probably has a different pattern, it is likely that it spends at least as much time under water as the captive seal.

Effects of "diving" and excitement

Since the seal actually spends as much as $\frac{1}{3}$ of its time diving one wonders if the renal function is invariably reduced to $\frac{1}{10}$ of its normal value during all forms of diving.

Unfortunately this question cannot be answered conclusively from the present study. The difficulty in obtaining clearcut results lies in the fact that fighting and struggling have a marked effect upon the renal function in the seal. Since most attempts to induce apnea resulted in some degree of struggle very few sufficiently long clearance periods were obtained in which apnea without struggle could be observed. It is evident from our observations (table 2) that apnea in connection with excitement caused a decrease in the GFR and a reduction in the urea U/P ratio. Excitement alone did

TABLE 2

	UREA	INULIN OR CREATININE	GFR
	U/P	*U/P*	*ml/min.*
Apnea without fighting	44.8	286	52.0
	29.0	337	51.6
Apnea with fighting	12.1	256	28.4
Struggle; no apnea	14.4	222	51.0
	21.9	217	69.0
	14.1	230	51.0
	15.8	331	54.0
	15.4	317	60.3
	11.0	167	73.0

TABLE 3

Electrocardiogram

	HEART RATE PER MIN.	T-WAVE	P-R INTERVAL
Normal breathing	120	normal	0.13
	150	normal	0.14
	155	normal	0.11
Apnea without fighting	29	normal	0.14
	12	normal	0.20
	22	normal	0.19
Apnea with fighting	48	inverted	0.14
	59	inverted	0.13
	56	isoeletric	0.24
	110	isoelectric	—
	59	diphasic	0.14

not cause a reduction in GFR but the urea U/P ratio fell significantly. Apnea not accompanied by struggle may or may not decrease the GFR. The question deserves further investigations.

The electrocardiograms obtained during the two different types of apnea were markedly different.[6] From table 3 it can be seen that during apnea induced by trickling water over the nose bradycardia is marked. The P–R interval is prolonged, but the T-waves show normal amplitude. In contrast to these findings the apnea accompanied by struggling, induced by holding an inverted closed funnel over the nostrils of the animal, did not produce as marked bradycardia and the T-waves became isoelectric or inverted.

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[6] No difficulties were encountered in obtaining electrocardiogram recordings during apnea not accompanied by struggle because no long periods were required.

DISCUSSION

The findings on the urea excretion in the fed and fasted seal show that the increase that occurs after feeding is apparently not due to a tubular regulation of the same nature as that found in sheep, man, and dog after prolonged changes in protein intake. The 50-fold increase in urea excretion that Smith found in seals after feeding can be accounted for by the increase in plasma urea concentration and in glomerular filtration rate and primarily by the increase in urine flow which might cause the urea/inulin clearance ratio to increase as much at 8 to 10-fold (see fig. 3).

It is noteworthy that the change in GFR that occurs after feeding apparently has no effect upon the fraction of filtered urea excreted. We do not as yet know in what manner the changes in GFR in the seal are brought about, i.e., if the filtration rate changes in all glomeruli or if there is an intermittency of function of nephrons (Page et al., '54). Since each kidney of a seal consists of approximately 80 separate renculi or units (Sperber, '44), it is possible that these complete units may show intermittency of function. The fact that lowering of GFR does not affect the concentrating ability of the kidney (Page et al., '54) lends some support to this latter suggestion.

The steepness of the curve for urea/inulin clearance ratio and the low maximum urea U/P indicate a rather low urea concentrating capacity in the seal kidney. The curve resembles that of man or dog on low protein intake (Murdaugh et al., '58; Schmidt-Nielsen, Murdaugh and O'Dell, in preparation), or of the premature human infant (Barnett et al., '48). In terms of the new hypothesis for the urea excretion mechanism proposed by Schmidt-Nielsen, ('58) such a finding may be interpreted as a low capacity of the renal tissue to transport urea. Further testing of this possibility would be valuable.

The finding that the urea U/P ratio is lowered in periods of struggle and excitement is in agreement with observations made in kangaroo rats and white rats. In these animals the fraction of filtered urea excreted became depressed during periods of excitement (Schmidt-Nielsen, '54). It seems very likely that the observations made by Page et al. ('54), on the lowering of the urea/creatinine clearance ratio during simulated diving is a manifestation of the same phenomenon, namely a result of struggle in connection with forced apnea rather than a response to diving and lowering of the filtration rate.

From the study of the electrocardiograms it is evident that the "diving" accompanied by struggle and fear had an effect upon the heart different from that of the quiet "diving." In the quiet "diving" each heart cycle was normal and the T-wave was not inverted. Scholander ('40) has reported that in his diving experiments with trained seals the electrocardiograms showed that the heart rate slowed down promptly during the diving to about 1/10 of the normal rate. In spite of the considerable decrease in rate each heart beat was normal or inconsiderably changed. It was thus observed that in the course of an 8 minute dive the P-R interval gradually increased from 0.25 to 0.30 seconds, but the T-wave was normal. Not before 15 to 18 minutes dive when the oxygen content of the blood had reached 3 volume per cent did the T-wave change and become inverted.[7]

We feel that on the basis of the marked differences in electrocardiogram during different types of "diving" the question concerning the effect of diving upon the GFR cannot be considered completely elucidated. It is evident that forced apnea in combination with struggling results in a reduction in the GFR but it is not evident to what extent natural diving affects the renal plasma flow and filtration rate.

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[7] In Scholander's experiments the seals were gradually trained over a long period to tolerate prolonged dives. Diving was carried out in a bathtub with the seal strapped to a board. It is apparent from Scholander's discussion that the seals struggled very little or not at all.

SUMMARY

Experiments on the effects of feeding and diving upon the renal function of the seal were carried out. It was found that the large increase in urea excretion which occurs in seals 3–5 hours after feeding is not due to renal tubular regulation of the urea excretion but can be accounted for by the increases in plasma urea concentration, glomerular filtration rate and urine flow. The urea/inulin clearance ratio in the seal decreases markedly with increasing tubular reabsorption of water. A maximum urea U/P ratio of 50–60 is reached already at an inulin U/P ratio of 150–200. Thus, the concentrating ability for urea is rather poor in the seal. A possible interpretation is that active transport of urea is not very pronounced in the renal tissue of the seal. The effect of apnea upon the renal function and upon the heart was found to depend upon the degree of struggling with which artificial "diving" was accompanied. Apnea not accompanied by struggle caused a slowing of the heart rate but each heart cycle was normal. In apnea accompanied by struggling the T-waves were inverted or isoelectric. When the animal was struggling, (with or without apnea), the maximum urea U/P ratio was decreased. It is not clear to what extent quiet diving affects the GFR.

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COMPARATIVE ENZYMATIC STUDIES ON CELLS OF TETRAHYMENA PYRIFORMIS Y FROM CULTURES AT TWO STAGES OF GROWTH: L-GLUTAMIC ACID DEHYDROGENASE

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TWO FIGURES

Although many studies have been made of possible physiological differences between cells from rapidly-dividing (logarithmic) cultures and from cultures in which cellular multiplication has slowed down or ceased, very little evidence regarding possible differences in chemical composition of cells in these two conditions can be found in the literature. Virtanen ('49) has reported that the activities of some constitutive enzymes of certain bacteria and yeasts do not vary with the state of nutrition of the cell; to our knowledge no similar comparative studies have yet been reported for protozoan or animal cells in axenic culture.

In a study of factors related to growth (mass cultures) of *Tetrahymena*, Phelps ('35) showed that the growth curve of these protozoan cells was similar to those of bacteria and yeasts in that the usual 5 phases of growth were recognized. However, in the case of the cells of *Tetrahymena* Phelps found that the change from logarithmic phase to the phase of negative growth acceleration was much more sharply marked than in bacterial and yeast cultures. Later Phelps ('36) reported that in axenic mass cultures of *Tetrahymena*, the yield of cells at the end of both logarithmic and stationary phases was directly proportional to the concentration of nutrient present in the medium. This indicated that the quantity of nutrient available was the limiting factor controlling growth. If limitation of growth, then, is nutritional, a quantitative study of possible chemical differences in cells harvested from mass cultures in the different growth phases should be of value as indicating differences in physiological state.

This paper is a report of assays for L-glutamic acid dehydrogenase activities of cells harvested from logarithmic and early mature cultures of *T. pyriformis* (Y). The presence of this enzyme in *Tetrahymena* had been demonstrated by Seaman ('51), and its important role in linking carbohydrate and nitrogen metabolism warranted its choice.

MATERIALS AND METHODS

Mass cultures of *T. pyriformis* Y were grown axenically in a medium containing the same concentrations of Proteose-Peptone and added inorganic salts as that used by Browning, Brittain and Bergendahl ('52). It also contained 0.05 M disodium phosphate and 0.1% (w/v) Difco yeast extract, and was adjusted to pH 7.3 with HCl acid before final dilution and autoclaving. The medium was autoclaved for 15 minutes at 15 lbs. per sq. inch pressure and was filtered to remove the small amount of amorphous precipitate which formed during this process. It was then measured into 1-liter flasks similar to those described by Browning, Brittain and Bergendahl ('52) and autoclaved a second time under the same conditions. After cooling, the medium was inoculated with 2 to 5 ml of a 24-hour culture of *T. pyriformis* Y which had been grown in a test-tube culture on the same medium; and 1.0 ml of a separately-prepared and autoclaved solution of thiamine hydrochloride was aseptically added per liter of medium. The cultures were incubated in a water bath at 37°C with aeration and in the dark.

Samples were taken within 10 minutes of inoculation, and thereafter at intervals of 4 to 6 hours during the day. A volume of fixative equal to the volume of the sample was added to each sample. The fixative contained 10% by volume of formalin in addition to all of the components of the medium (except the yeast extract and the thiamine); the pH of the fixative was readjusted to 7.3 after the addition of the formalin. Cell counts were made on the fixed cell suspensions; a Sedgewick-Rafter chamber was used with a microscope equipped with a calibrated Whipple eyepiece micrometer. The cells in 100 microscopic fields were counted in each sample; the later, more populous samples of each culture were diluted so that approximately 500 cells were counted.

Approximately 20 hours after inoculation, while the culture was still in or near the "logarithmic" phase of growth, the cells from 500 ml of the culture were harvested. The 500 ml of culture was distributed in 50 ml centrifuge tubes, which were chilled in an ice bath for about 20 minutes, and were then centrifuged for 4 minutes at approximately 90 × g (to avoid damage to the cells) at 0°C in a refrigerated centrifuge. The supernatant fluid was decanted, and the cells were resuspended in a cold mineral salts solution. Since washing the cells with distilled water caused extensive lysis, it could not be used. The salt solution contained the same salts in the same concentration as did the medium, and like the medium was 0.05 M in disodium hydrogen phosphate and had been adjusted to pH 7.3 with hydrochloric acid. The cells were centrifuged and washed three times, pooled in a total volume of about 10 ml, and transferred to a homogenizer tube which fitted the centrifuge head.

A 0.1-ml sample of the concentrated washed cell suspension diluted with the formalin-containing fixative was used for cell counts; duplicate 1-ml samples were used for the determination of total N by the micro-Kjeldahl method. The concentrated washed cell suspension was then centrifuged in the cold for 6 minutes at approximately 600 × g, and the supernatant was discarded from the 1 to 2 ml of packed cells which remained. The cells were homogenized in the cold, and samples of the homogenates were used for the determination of L-glutamic acid dehydrogenase activity, according to the method of Wingo and Thompson ('56). Other samples of the homogenate were subjected to the micro-Kjeldahl determination of total nitrogen. From the various data, the micrograms of ammonia nitrogen liberated in 20 minutes per 10^5 cells and per milligram of total protozoan nitrogen were calculated.

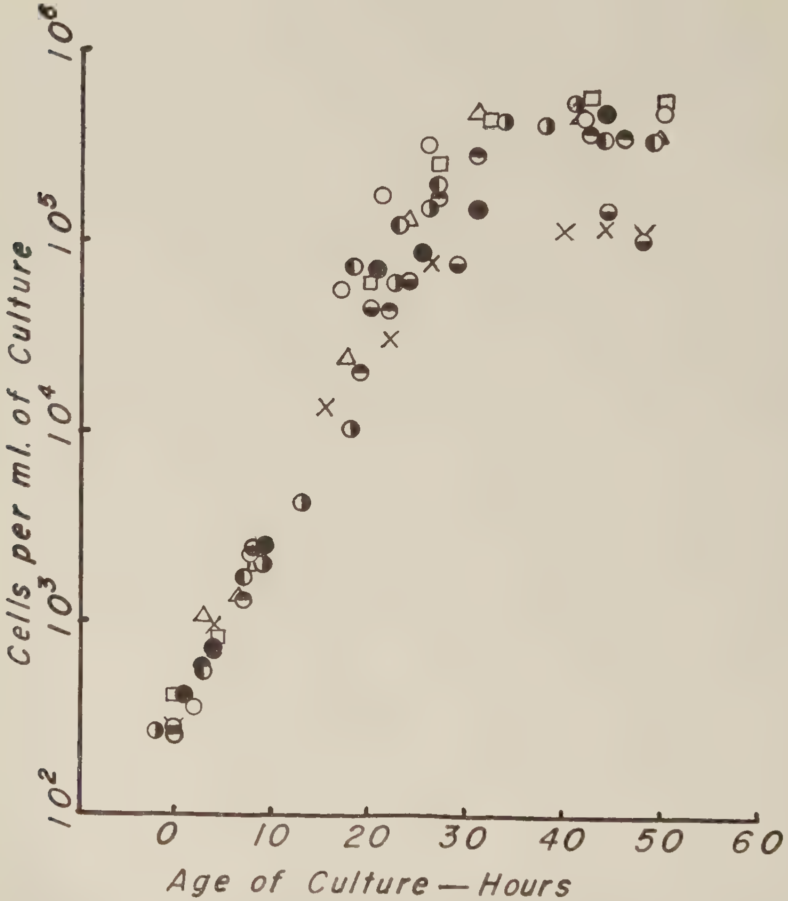

Fig. 1 Growth of *T. pyriformis* Y cultures assayed for L-glutamic acid dehydrogenase.

Key to run numbers: Run 1, ○; 2, ◐; 3, ◑; 4, □; 5, ●; 6, ×; 7, △; 8, ◓; 9, ◒.

After the removal of the 500 ml for use in the first harvest and assay, the growth of the culture remaining in the flask was continued, as were the cell counts. When the stationary phase of growth had been attained or closely approached, the cells in 150 to 200 ml of the culture were harvested and subjected to assay in the manner described above.

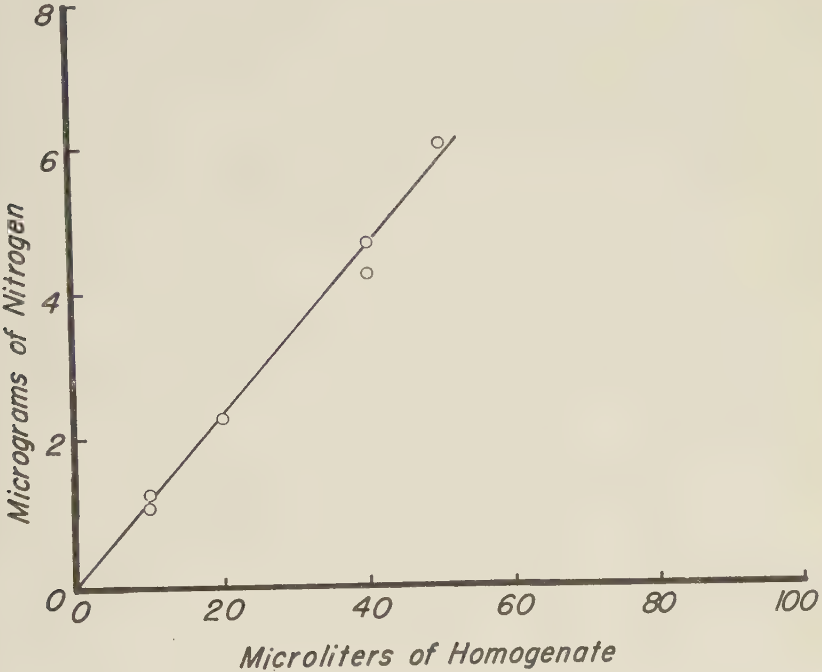

Fig. 2 Activity of L-glutamic acid dehydrogenase in homogenates of *T. pyriformis* Y.

RESULTS

Growth rates

Figure 1 is a composite time-population plot showing growth of 9 separate cultures; the enzyme content of each culture was determined at two separate times. In general, the behavior of the various cultures was very similar; cultures 6 and 9 failed to attain the maximum populations reached by the other 7 cultures. This probably occurred because of slight variations in preparation of the medium, especially in rate of cooling of the autoclave.

Assay for L-glutamic acid dehydrogenase

Each determination of L-glutamic acid dehydrogenase activity was carried out using at least three levels of addition of homogenate to the assay system, and in each case excellent proportionality of response to dose was obtained. As an example, a plot of this relation for one assay has been recorded in figure 2.

TABLE 1

L-glutamic acid dehydrogenase activity of Tetrahymena pyriformis Y

RUN	LOG PHASE			STATIONARY PHASE		
	Age of culture [1]	μg N/10^5 cells/ 20 min.	μg N/mg total protozoan N/20 min.	Age of culture [1]	μg N/10^5 cells/ 20 min.	μg N/mg total protozoan N/20 min.
	hrs.			*hrs.*		
1	21	0.37	50.4	46	0.87	50.8
2	23	0.36	39.6	44	0.90	39.8
3	22	0.32	37.6	38	0.95	48.6
4	21	0.42	45.3	42	0.65	32.7
5	21	0.28	34.6	44	0.85	48.1
6	22	0.45	29.8	44	1.08	36.6
7	24	0.55	24.6	45	2.55	31.7
8	22	0.73	62.9	41	1.18	48.6
9	24	0.45	40.6	44	0.87	41.7
Averages		0.45	40.6		1.10	42.1

[1] Corrected to equal times at population of 1000 cells/ml.

The results obtained from the 9 cultures are shown in table 1. Seaman ('51) determined the L-glutamic acid dehydrogenase activity of *T. pyriformis* S, grown for 48 hours at 25°C. This was equivalent to the uptake of 3.3 μl of O_2 per milligram dry weight of cells per hour; since he found the dry weight of 10^6 cells to be 14.3 mg, the oxygen-uptake observed was equivalent to 1.96 μg N per 10^5 cells per 20 minutes. In view of the differences in strain of organism and in culture conditions used, the agreement between his value and the average value of 1.10 μg N per 10^5 cells per 20 minutes from our older cultures may be regarded as satisfactory.

DISCUSSION

The assays for L-glutamic acid dehydrogenase activity give an average of 0.45 μg N per 10^5 cells per 20 minutes on cultures in the logarithmic phase and an average of 1.10 μg N per 10^5 cells per 20 minutes on cultures in the early mature phase. Application of the "t" test to these average values for enzymatic activity of the young and mature cultures expressed on a cellular basis gives a value of 3.36. A "t" of 3.85 is obtained when the test as modified for paired data is applied. Both of these values indicate that the difference between young and mature cultures is highly significant. However, as Wingo, Lockingen and Cameron ('55) have reported, the average size of the cells becomes larger as the culture approaches maturity. The differences in enzyme content, calculated on a cellular basis, are possibly due to this difference in cell size.

A comparison of the cells from young and mature on a weight basis would probably be more nearly valid than the above comparison on a number basis. The weight of the tissue used could not be found from dry-weight determinations since a salt solution was of necessity used to wash the cells. The total nitrogen content of the homogenates should be a valid index of their content of protoplasm. The results have therefore been expressed on the basis of enzyme activity per mg total protozoan nitrogen. Averages obtained thus, expressing activities on the basis of total nitrogen content, are 40.6 μg N per milligram total protozoan N per 20 minutes for the cultures in log phase and 42.1 μg N per milligram total protozoan N per 20 minutes for the cultures in stationary phase. The "t" for these data, is 0.33; the corresponding paired-value "t" is 0.485. Both of these tests indicate that the difference between the enzyme concentrations of cells harvested from young and mature cultures is negligible.

It can be inferred from this finding that the physiological state of the protoplasm of cells from early mature cultures does not differ significantly from that of cells from rapidly-growing cultures, and that cellular multiplication in *T. pyriformis* is terminated by exhaustion of nutrient.

SUMMARY

Bacteria-free mass cultures of *Tetrahymena pyriformis* were grown in modified Phelps medium. Cells from cultures in the logarithmic and early mature phases of growth were harvested by centrifuging and washing. Homogenates of these cells were assayed for L-glutamic acid dehydrogenase activity by the authors' microdiffusion method. Per cell, the cells from mature cultures contained more enzyme than did those from logarithmic cultures; but enzymatic activity per milligram of total protozoan nitrogen in cells harvested from the two phases did not differ significantly. These results indicate that the termination of cellular multiplication is not necessarily accompanied by a change in physiological state of the cells.

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SOME PROPERTIES OF CULTURED CHICK SKELETAL MUSCLE WITH PARTICULAR REFERENCE TO FIBRILLATION POTENTIAL

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ELEVEN FIGURES

INTRODUCTION

A previous communication (Li, Shy and Wells, '57), reported that spontaneous fibrillation potentials were recorded with intracellular electrodes from rat skeletal muscle fibers two days after denervation. Since there was no assurance in the placement of the recording electrode, the possibility of this spontaneous activity being initiated in the end-plate zones (Hayes and Woolsey, '42) remained.

Skeletal muscle fibers in tissue culture explants show spontaneous rhythmic contractions (Lewis, '15; de Renyi and Hogue, '34; Inditskaia, '38; Szepsenwol, '42; Pogogeff, '46; Pomerat, '53), resembling, in many respects, the fibrillary movement observed in denervated skeletal muscle fibers *in vivo.* An attempt to record the electrical activity from these muscle fibers was made by Szepsenwol ('46). With extracellular electrodes he demonstrated spontaneous rhythmic potentials from groups of contracting muscle fibers in tisue explants, but met difficulty in studying single muscle fibers which he attributed to the "smallness of the current generated by a single cell."

Recently, Crain ('56) was able to show that action potentials of 95 mv could be recorded from the interior of a spinal ganglion cell of the chick in tissue culture explants. Furthermore, in view of the similarities in the electrical responses to stimulation between the cultured ganglion cells and various types of neurones *in vivo,* Crain suggested that when properly maintained the cultured tissue could retain many of its normal functional characteristics.

The present investigation concerns the spontaneous electrical activity of chick skeletal muscle fibers grown in tissue culture. It will be shown that spontaneous rhythmic potentials identical to those observed in denervated muscle fibers *in vivo* were recorded with intracellular electrodes from the cultured muscle cells. Since neuronal elements and end-plate organs were not found in the tissue culture explants, a conclusion can be drawn that the spontaneous rhythmic potentials were not originated in the end-plate zones. The results of this study also permit some deductions to be made in regard to the mechanism for the production of spontaneous rhythmic discharges. Finally, different types of muscle responses to stimulation were found, suggesting that a functional differentiation of skeletal muscle might exist as early as the 7th day *in vitro.*

METHOD

Tissue cultures

Primary explants of skeletal muscle from the thighs of 13-day-old chick embryo were cultivated in either a plasma clot or reconstituted rat tail collagen (Bornstein, '58) on flying coverslips. They were placed in roller tubes containing 2 ml of feeding solution composed of 50% horse serum, 5% 9-day-old chick embryo extract, and 45% modified Gey's balanced salt solution (BSS) (Gey, G. and Gey, M., '36). The latter consisted of $NaCl$, 0.800; KCl, 0.037; $CaCl_2 \cdot 2H_2O$, 0.017; $MgCl_2 \cdot 6H_2O$, 0.021; Na_2HPO_4, 0.012; KH_2PO_4, 0.003; $NgSO_4 \cdot 7H_2O$, 0.007; $NaHCO_3$, 0.023 and glucose, 0.600% (w/v) in 100 ml of aqueous solution; to this solution was added 10,000 units of penicillin "G" sodium and 13.5 mg of streptomycin sulfate. The roller tubes were put in compartments of a drum which revolved once every minute at a constant temperature of 37°C.

The feeding solution was changed depending on its pH value, usually once every 2–3 days. The electrical activity of the tissue was studied 7–21 days after explantation.

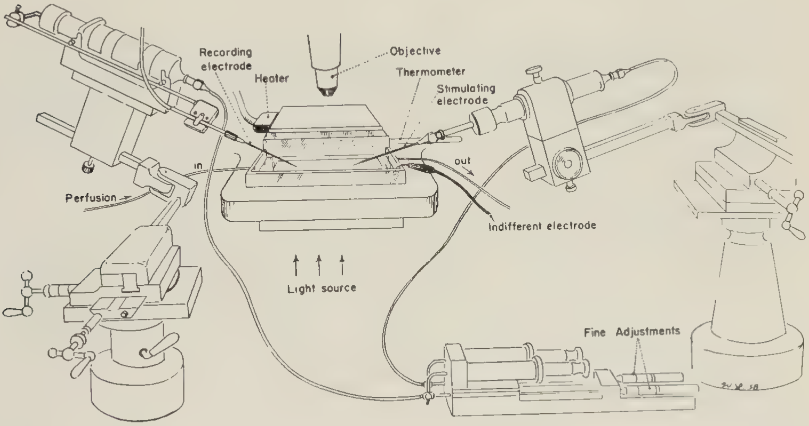

Fig. 1 A schematic drawing of the tissue chamber under the microscope and the arrangement of the recording and stimulating electrodes.

Tissue chamber

The chamber (fig. 1) consisted of a shallow rectangular "Plexiglas" vessel (3.0 × 3.5 × 0.3 cm) mounted on a heating stage, the temperature of which could be accurately regulated. On the two sides of the vessel were openings connected to flexible tubing through which a slow continuous perfusion of the BSS into the chamber could be established when required. The volume of the solution in the chamber was regulated to be under 2–3 ml. The tissue chamber was attached to the mechanical stage of a binocular microscope provided with an attachment for microphotography (fig. 2).

With the solution in the chamber constantly at 37°C, the coverslip with the tissue explants was transferred from the roller tube to the tissue chamber. A single stimulating pulse of 5 v and 0.7 msec. applied to the tissue was usually adequate to evoke a response from the muscle in the chamber. An increase in the response threshold during the experiment signified the need for a change of either the solution or the tissue culture explant. In most instances, tissues in the chamber continued to respond to stimuli of similar strength for as long as 4 hours after removal from the roller tubes.

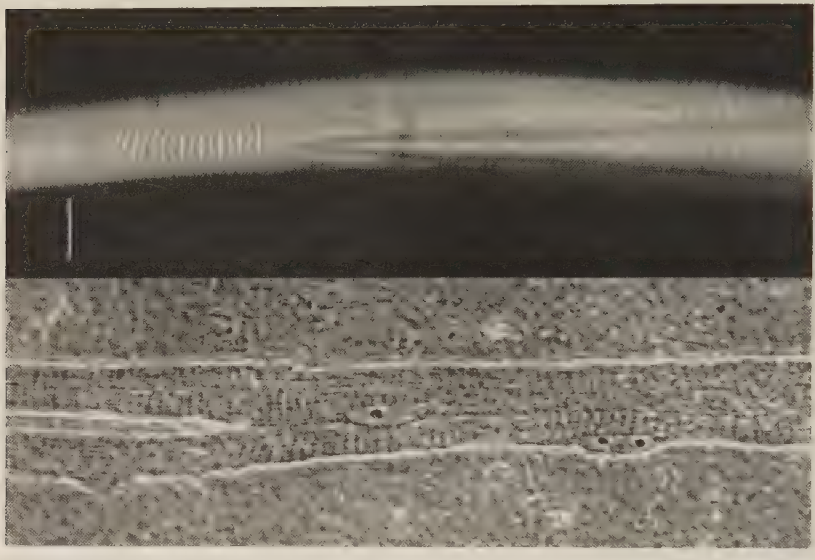

Fig. 2 Microphotographs showing (upper) a living embryonic muscle cell and glass micropipette in polarized light and (lower) a similar cell in phase contrast illumination. Vertical bar represents 10 μ.

Recording of muscle potentials and electrical stimulation

Glass micropipettes filled with 3 M KCl agar agar and measuring less than 0.2 μ in outside diameter were used for recording. The d.c. resistance of these electrodes ranged between 10 and 30 megohms. The reference electrode consisted of an agar agar bridge in the tissue chamber. The recording system essentially resembled that which has been previously described (Li and McIlwain, '57). The stimulating electrodes consisted of two tungsten wires, tapered and insulated except at the tips according to the method of Hubel ('57). The d.c.

resistance of the tungsten stimulating electrodes measured 1–2 megohms.

RESULTS

Extracellular records

Although electrical activity could be recorded from cultured muscle fibers two days after explantation, consistent results were more readily obtained from the 7th to 21st day.

1. Spontaneous rhythmic activity. When the microelectrode was placed near the twitching muscle fibers, spontaneous rhythmic activity could be recorded. The rhythmic potentials occurred in bursts synchronous with the rhythmic movement of the muscle observed through the microscope. In the experiment shown in figure 3A, each burst consisted of 6 waves approximately 100 msec. in duration, and the interpotential intervals ranged between 80 and 110 msec. Between the bursts were silent periods varying from three seconds to several minutes.

The duration of active periods and the configuration of the waves in a burst were not always constant. Figure 3B shows that a burst of the rhythmic activity would persist for as long as 5–7 seconds and the shape and size of the waves in a given burst would change. At times, potentials of relatively consistent form were recorded (fig. 3C). In any case, the frequency of the rhythmic waves recorded with extracellular electrodes from any given fiber was fairly constant, being between 6 and 13 per second.

It is to be noted that the twitching movements of the muscle fibers observed through the microscope were not always synchronous. In fact, asynchronization of movement was more frequently seen and even occurred in muscle tissue which also exhibited synchronous twitchings. At times synchronous slow contraction and prolonged relaxation of a number of muscle fibers were observed in a tissue explant where many of the muscle fibers underwent twitching movements. There were also occasions on which twitching movements began in one or two fibers and rapidly spread resulting in synchronous

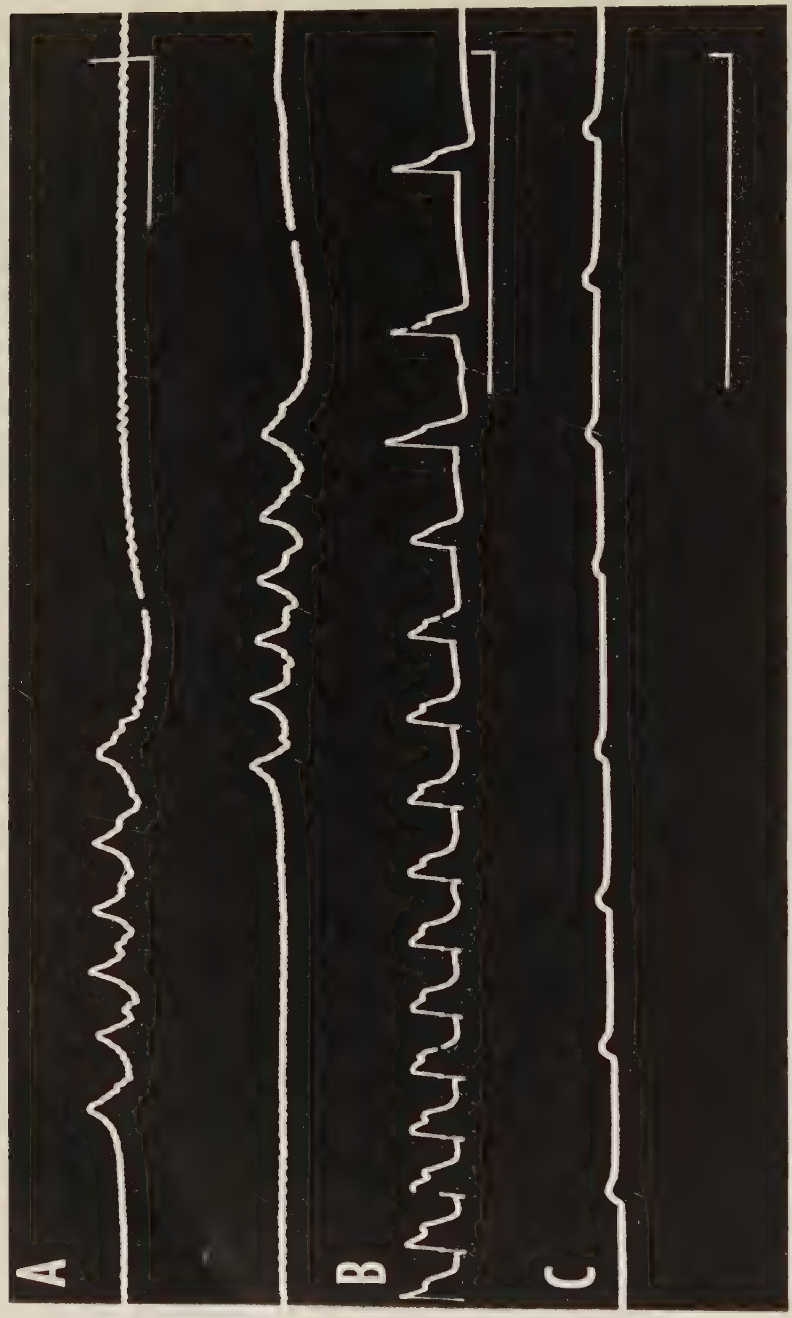

Fig. 3 Spontaneous rhythmic potentials recorded with extracellular electrodes. Vertical bars represent 2 mv; horizontal bars, 200 msec. In this and subsequent figures, positivity is upward.

twitching of many fibers in a considerable area of the tissue explant.

The variability in the configuration of the spontaneous rhythmic waves recorded in a volume conductor was probably due to the random orientation and varying speeds of conduction of the muscle fibers. The asynchronous movement of muscle fibers found at a distance from the recording electrode might also have been a factor. The increase or decrease in amplitude of the waves (fig. 3B) was considered to be due to an increase or decrease in the number of active cells in the area where the extracellular electrode was placed.

2. Response to stimulation. When stimulation was applied the responses of the muscle differed in form. They were monophasic positive (fig. 4A), negative-positive (fig. 4B) or monophasic negative (fig. 4C), varying in all likelihood according to the orientation of the activated fibers and the recording electrode. In figure 4A, recruitment of active fibers during repetitive stimulation could be seen. In this experiment, the muscle tissue was stimulated at a rate of 1/sec. and responses of increased size were recorded.

Stimulation at frequencies higher than 2–3/sec. usually caused a sustained contraction and prolonged relaxation of the muscle. At other times it would produce a twitch followed by a prolonged post-excitatory depression of the muscle. In both circumstances attempts to determine the time required for the muscle to recover from a single twitch usually proved unfruitful. Only on two occasions could responses to twin shocks be obtained for a long duration; the recovery period was found to be 30 and 33 msec. An example from one experiment is shown in figure 4D.

Intracellular potentials

1. Spontaneous rhythmic potential. When a microelectrode was brought into contact with an apparently inactive muscle fiber and instantly withdrawn, a series of fibrillary twitches could be produced apparently resulting from mechanical stimulation. The fibrillary movements frequently extended so that fibers in the adjacent area would twitch synchronously. If the microelectrode was inserted, usually it would either become dislodged within one or two minutes or

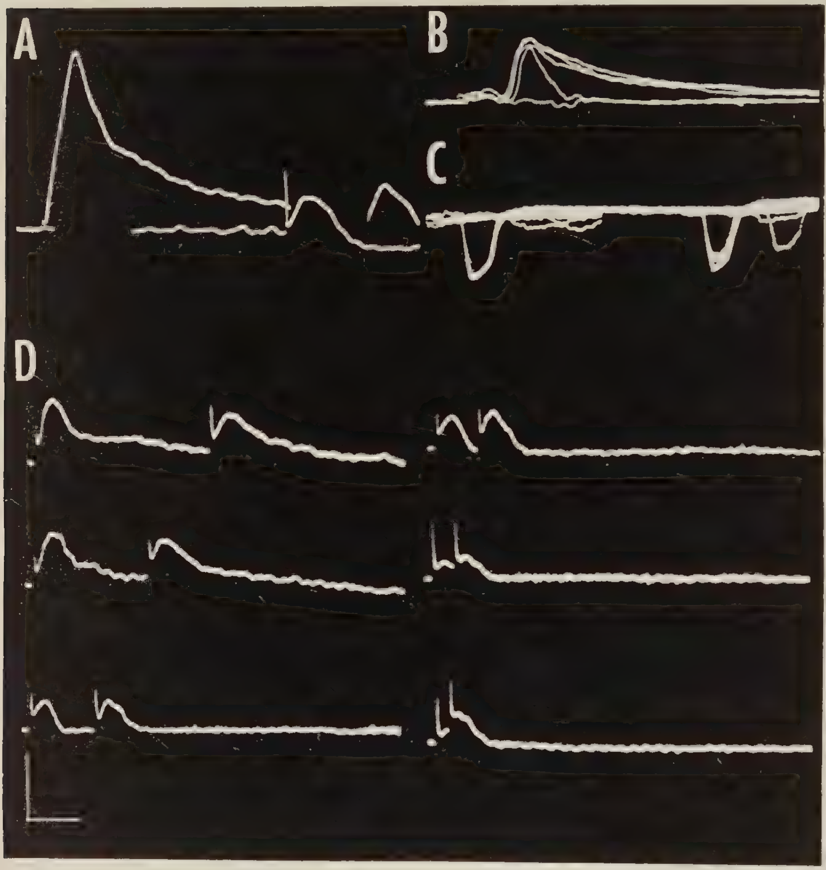

Fig. 4 Responses to electrical stimulation and recovery period recorded with extracellular electrodes. (A) A large monophasic positive potential occasionally occurred following stimulation at one per second. (B) In three of the 5 superimposed records the responses consist of an initially negative potential of about 0.5 mv and 40 msec. followed by a positive potential of about 2.2 mv and 500 msec. (C) Superimposed records of approximately 20 responses consisting of predominantly negative potentials. (D) Responses to twin shocks at intervals of 270, 175, 100, 60, 33 and 25 msec. Vertical bar represents 2 mv; horizontal bar, 100 msec.

cause irreparable damage to the fiber as indicated by rapid disappearance of fibrillary movement and gradual decrease of the recorded resting potential.

There were occasions on which no apparent disturbance to the muscle fiber was observed after the electrode had been inserted. The penetrating electrode would abruptly record a negative potential which might or might not be accompanied by initial injury discharges. The recorded negative potential would remain stable for as long as 30 minutes. However, if a burst of spontaneous movement should occur, the impaled electrode would again be dislodged or induce a rapid deterioration of the muscle fiber resulting in a return of the steady potential to its zero potential level.

Examples of the spontaneous rhythmic activity are shown in figures 5 and 6. In the experiment shown in figure 5A, the penetrating electrode recorded a negative potential of —75 mv which remained steady for about 30 seconds before spontaneous spike discharges suddenly occurred. The record shows moderate constancy of interspike intervals ranging from 100 to 108 msec. and the presence of a critical level of depolarization measuring 24 mv. In the experiments where the impaling electrode was not dislodged by the contracting muscle fiber, there was a progressive diminution of spike amplitudes and a replacement of spike potentials by spontaneous rhythmic oscillations (fig. 5B). The frequency of the oscillating potentials, however, was exactly the same as that of the spike discharges. The regularity of the spike frequency, the presence of a critical depolarization level, and the occurrence of rhythmic oscillations of potential in the absence of spike discharge were also observed in denervated mammalian skeletal muscle fibers (Li et al., '57). It was suggested that the spike is probably not a necessary link in the event leading to the rhythmicity (Li et al., '57); and its occurrence probably depends upon the rhythm and magnitude of the oscillating potentials (Li, '59).

The dependence of spike activity on the rhythmic oscillations of the membrane is further indicated by the continuous

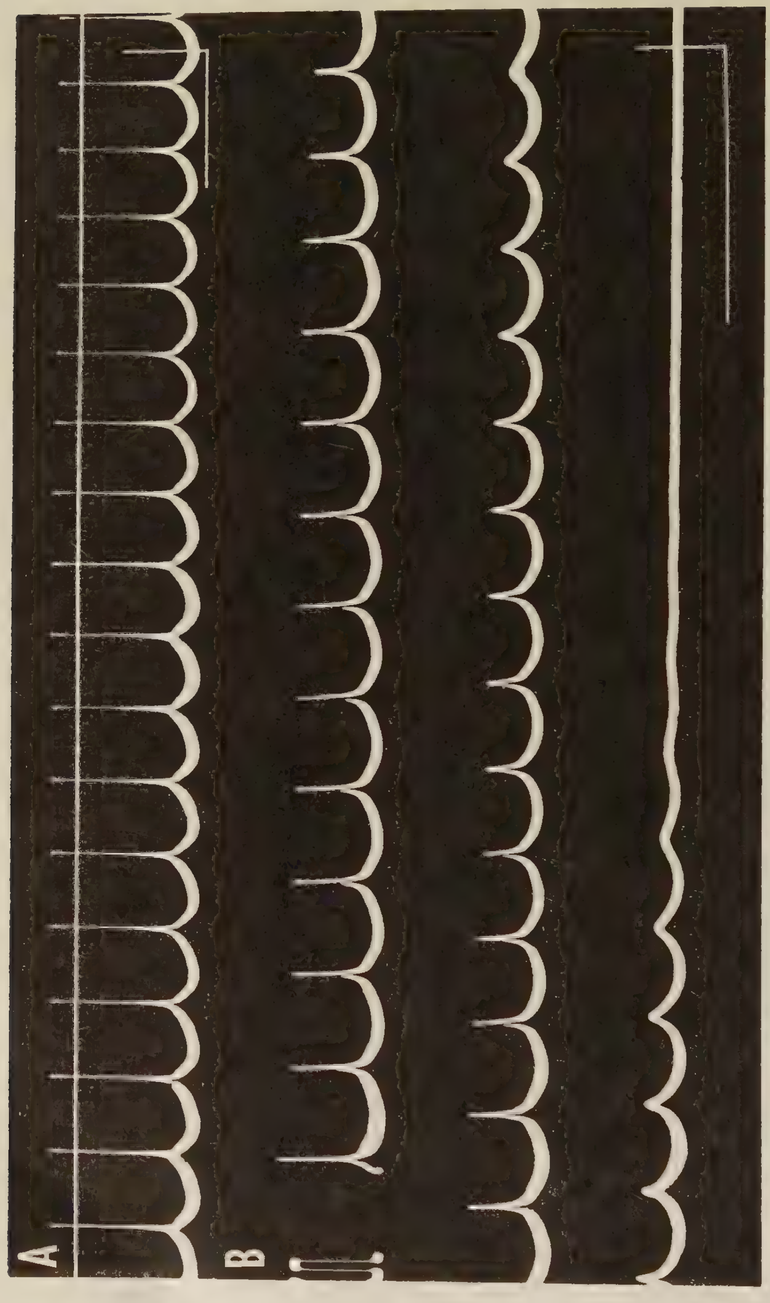

Fig. 5 Spontaneous rhythmic discharges recorded with intracellular electrodes from two muscle cells. First line in (A) represents zero potential; vertical bars represent 50 mv; horizontal bars, 200 msec.

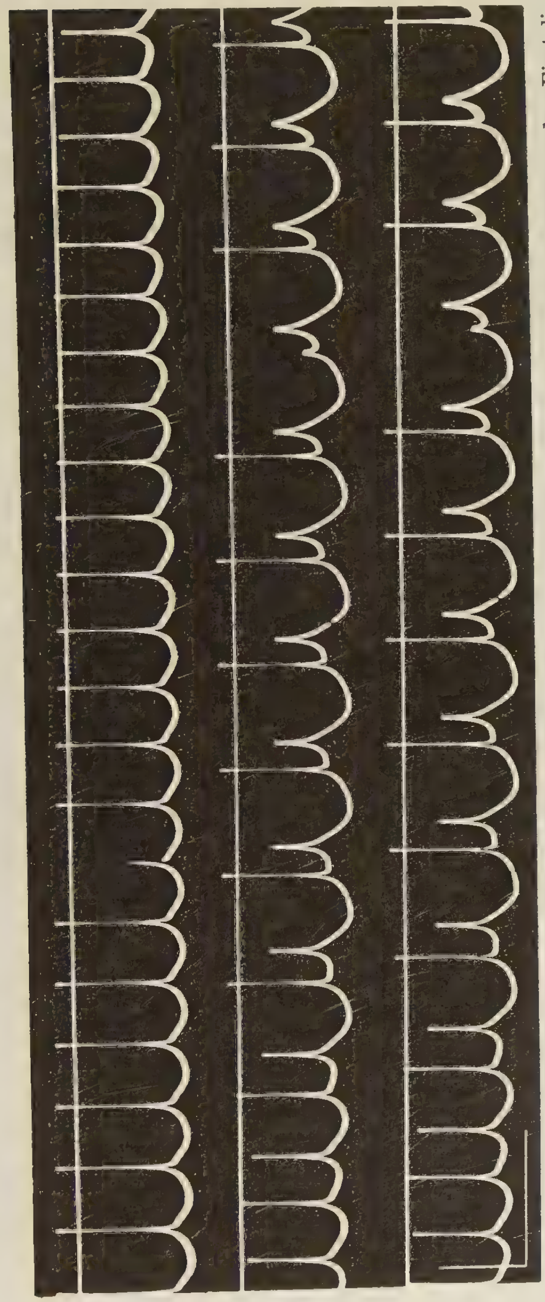

Fig. 6 Dissociation of spontaneous rhythmic spike discharges and oscillations of potential. Continuous records. First line represents zero potential; vertical bar, 50 mv and horizontal bar, 200 msec.

record shown in figure 6. This figure shows spontaneous rhythmic spike discharges with a resting membrane potential of —66 mv and critical depolarization of 14 mv. The interspike intervals measured from 78 to 84 msec. In the second and third rows of the record a dissociation of oscillating potentials and spike discharges can be seen. The frequency of the oscillating potentials became slower with interpotential intervals then measuring 150–160 msec. When an oscillating potential surpassed the level of 14 mv, a spike discharge occurred. However, despite the spike discharge, the oscillating potential continued to grow, reaching its usual time course of 150–160 msec. to arrive at its summit, and afterwards subsiding. The interval between the onset of the spike and the summit of the oscillating potential measured 30–35 msec.; this time interval corresponded to the refractory periods of muscle fibers obtained from extracellular records (fig. 4D). Thus, even though the height of the summit of the oscillating potential had far exceeded the critical depolarization potential, spike discharge either failed to occur or appeared with a much smaller amplitude. The occurrence of a spike discharge in the developing phase of an oscillating potential did not disturb the natural rhythm of the oscillating potential.

2. Response to stimulation. Single shocks applied to the muscle fiber evoked responses of various sizes and shapes. They could be classified into (a) twitch responses (fig. 7A) and (b) slow responses (fig. 7B, C, and D). The muscle fibers which produced different responses did not differ in their gross appearance except that following the stimulation one twitched and the other slowly contracted. Although the two types of responses could be obtained from fibers in the same explant, the twitch responses were more frequently noted. There was no indication of any relationship between the type of response and the age of the culture studied, which ran from 7 to 21 days.

A twitch response consisted of a spike potential with an amplitude sometimes as large as 100 mv and a duration of 2.0 msec. It was usually followed by a prolonged after-poten-

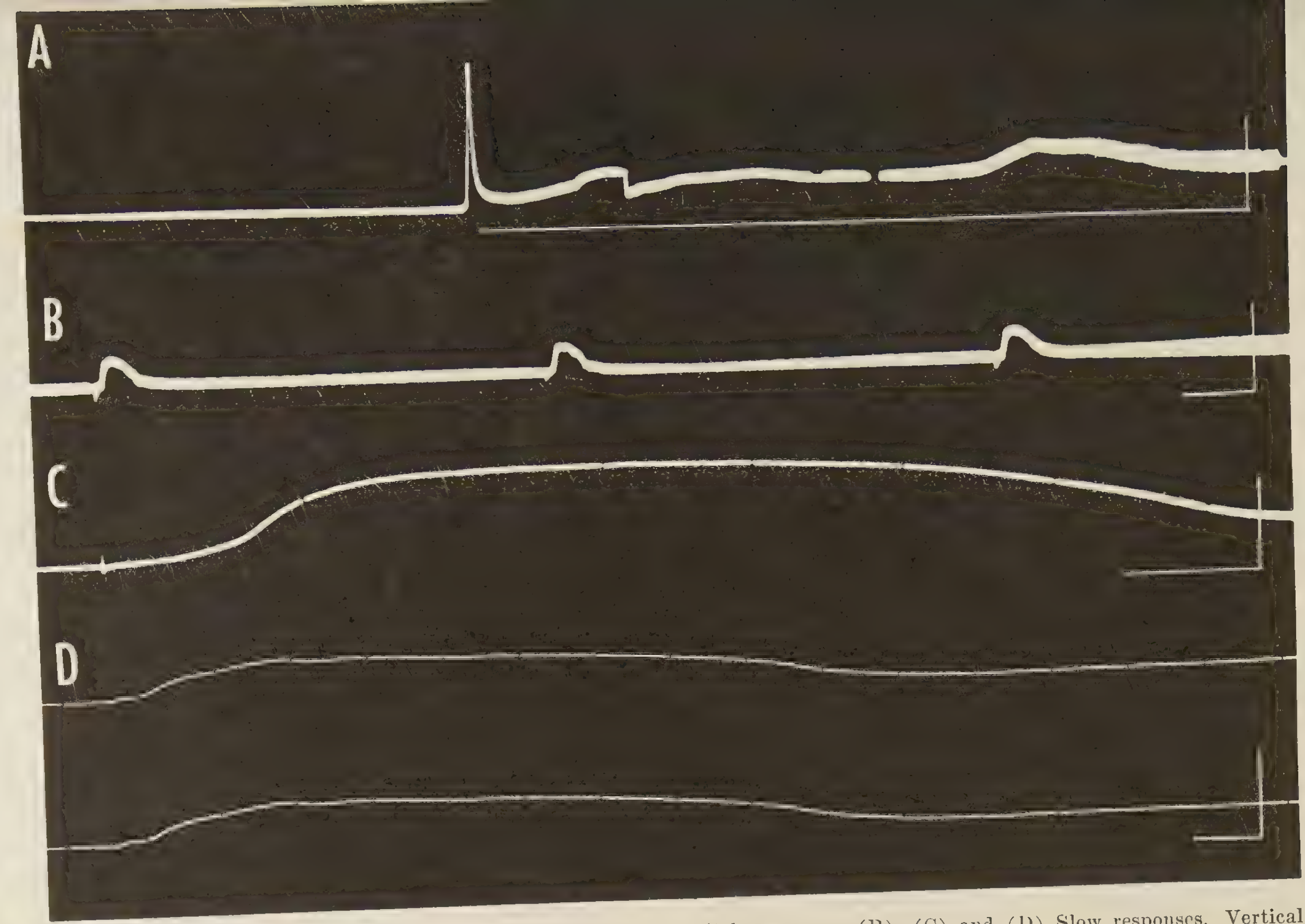

Fig. 7 Various types of responses to single shocks. (A) Twitch response. (B), (C) and (D) Slow responses. Vertical bars represent 50 mv; horizontal bars, 200 msec.

tial which might persist for as long as 1.8 sec. (fig. 8). Frequently, superimposed on the after-potential were oscillations similar to the rhythmic oscillating potentials described above. There were cases in which multiple spike discharges in response to a single stimulus were recorded. In these cases the recording electrode invariably became dislodged following the stimulation.

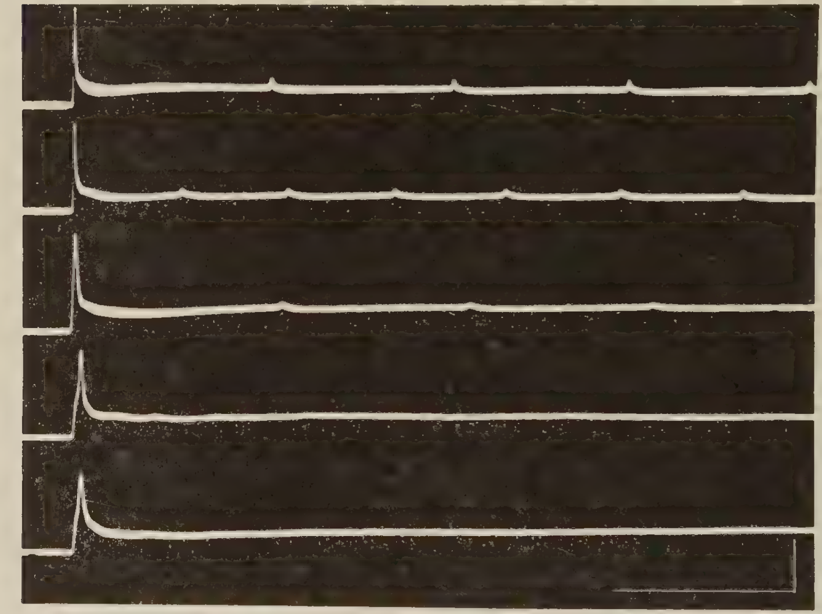

Fig. 8 Twitch responses following repetitive stimulation at frequency of about once every 10 sec. First line represents zero potentials; vertical bar, 50 mv; horizontal bar, 200 msec.

Figure 8 illustrates a twitch response recorded from a fiber stimulated approximately once every 10 seconds. In this experiment 20 responses were elicited in a course of three minutes and a progressive change in responses was observed. The spike amplitude decreased with an increase in spike duration. The oscillations of potential, which occurred 120 and 200 msec. after the spike discharges, also underwent a similar change. During the change of the spike from 97 mv and 2.0 msec. to 70 mv and 6 msec., the oscillating potentials disappeared. The resting membrane potential also decreased from —81 to —68 mv. There was an "inflection" at 37 mv depolarization in the rising phase of the spike. These changes were similar to those observed in cultured spinal ganglion cells (Crain, '56) most likely due to the deterioration of the cell membrane caused by the penetration of the microelectrode.

Occasionally, stimulation of a muscle fiber evoked a small response which, upon attaining a certain size, set off a spike discharge. An example is given in figure 9. In this experiment stimulation at 4/sec. elicited potentials which measured 22 mv and 10 msec. When the stimulation frequency was reduced to 3/sec., spike responses were obtained. The declining phase of the spike at the level of 31 mv depolarization was complicated by a "hump" which lengthened the total time course of the spike to 10 msec.; this duration was precisely the same as that of the small responses. The critical depolarization level in this fiber was probably between 22 and 31 mv. A "hump" in the falling phase of a response was seen in action potentials recorded from frog skeletal muscle fibers (Fatt and Katz, '51) cardiac muscle fibers (Weidmann, '52) and cultured spinal ganglion cells of the chick (Crain, '56). In those cases, the "hump" usually occurred at about the zero potential level and in no instance was it recorded in the absence of spike discharge. A local potential has been produced in partially curarized muscle fibers following nerve stimulation and in nerve fibers following a stimulus of strength inadequate to evoke a spike response; but if a spike potential was evoked, the local potential was curtailed. In the present experiment the small potential was recorded in the absence of spike discharge and its occurrence was not abolished by the spike. This might be a characteristic of the chick muscle fiber in cultured explants.

In attempting to determine the refractory period by applying twin shocks to the muscle fiber, difficulties similar to those

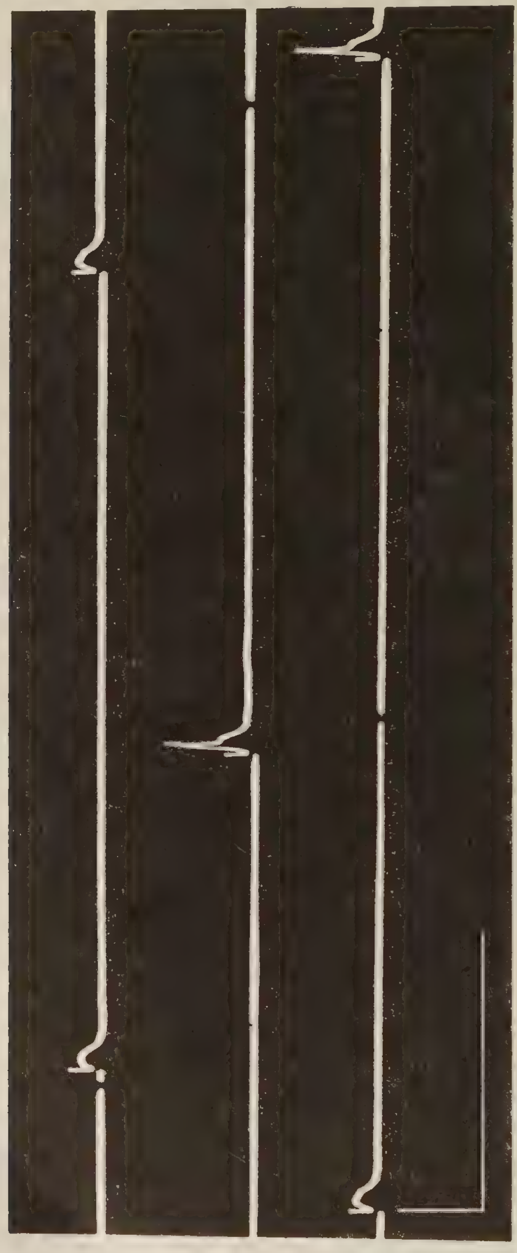

Fig. 9 Small potentials in response to stimuli at 4/sec. in (A) and 3/sec. in (B). Vertical bar represents 50 mv; horizontal bar, 80 msec.

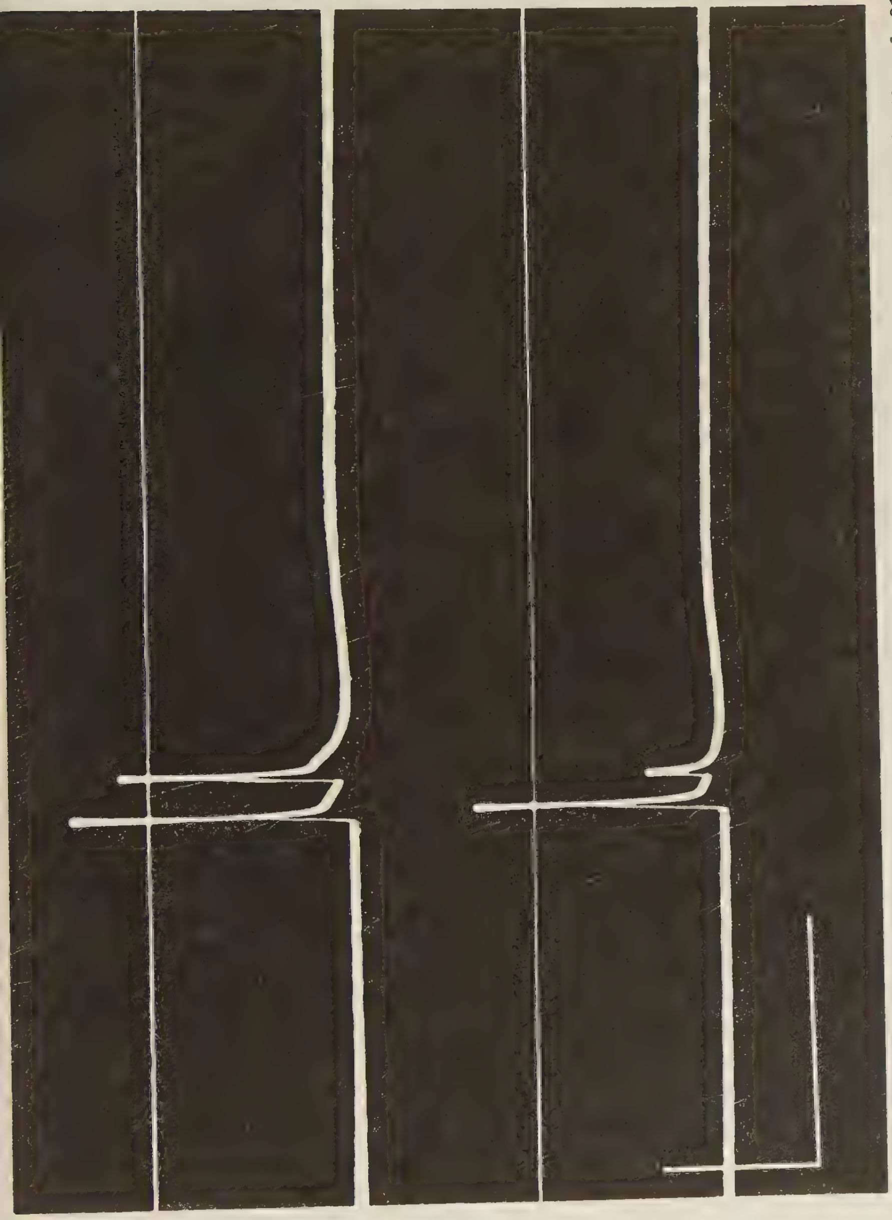

Fig. 10 Recovery period of a muscle cell with twitch responses. Intervals between paired stimuli, 40 and 31 msec. First line represents zero potential; vertical bar, 50 mv; horizontal bar, 200 msec.

described in the section on extracellular records were encountered. In three experiments the refractory periods were found to be 25, 31 and 33 msec. (fig. 10). These measurements were consistent with those recorded with extracellular electrodes.

The *slow responses* (fig. 7B, C and D) recorded from a given muscle fiber were fairly constant in size and shape, although variations were found from one fiber to another. In figure 7B, stimuli at 1/sec. were applied and 16 responses were recorded. In response to stimulation, the potential slowly rose to a peak of 20 mv in 10 msec., and after remaining at this level for about 25 msec., gradually fell. In its falling phase, there were small and irregular fluctuations. The whole event took approximately 100 msec. In the experiment shown in figure 7C, a single stimulus produced a slow depolarization to 14 mv in 200 msec.; the potential then rose with a relatively faster speed to reach 48 mv and remained there for about 800 msec. before repolarization took place. The total time course of the response measured 1,625 msec.

The duration of the slow responses recorded from another fiber was found to be even longer (fig. 7D). In this fiber a stimulus elicited an irregular and slow rise of potential to a plateau of 22 mv in 400 msec., where it hovered for some 2,000 msec. before declining to its original level. The whole response lasted for 2,960 msec. Through the microscope the muscle showed a slow irregular contraction followed by slow relaxation.

Paired stimuli of identical strength applied at various intervals to fibers producing slow responses revealed that the refractory periods of these fibers would last for several seconds. Figure 11 was taken from the same experiment shown in figure 7C. The refractory period of this fiber was found to persist for 4.2 seconds.

In some respects, the slow responses were similar to the contracture activity described by Bremer ('32) and Kuffler and Vaughan Williams ('53). Similar potentials were also recorded from the frog (Li and Gouras, '57) and rat (Li, '58) skeletal muscle fibers subjected to cold temperatures. In the present investigation, the existence of fibers with twitch responses and slow responses in the same tissue explant sug-

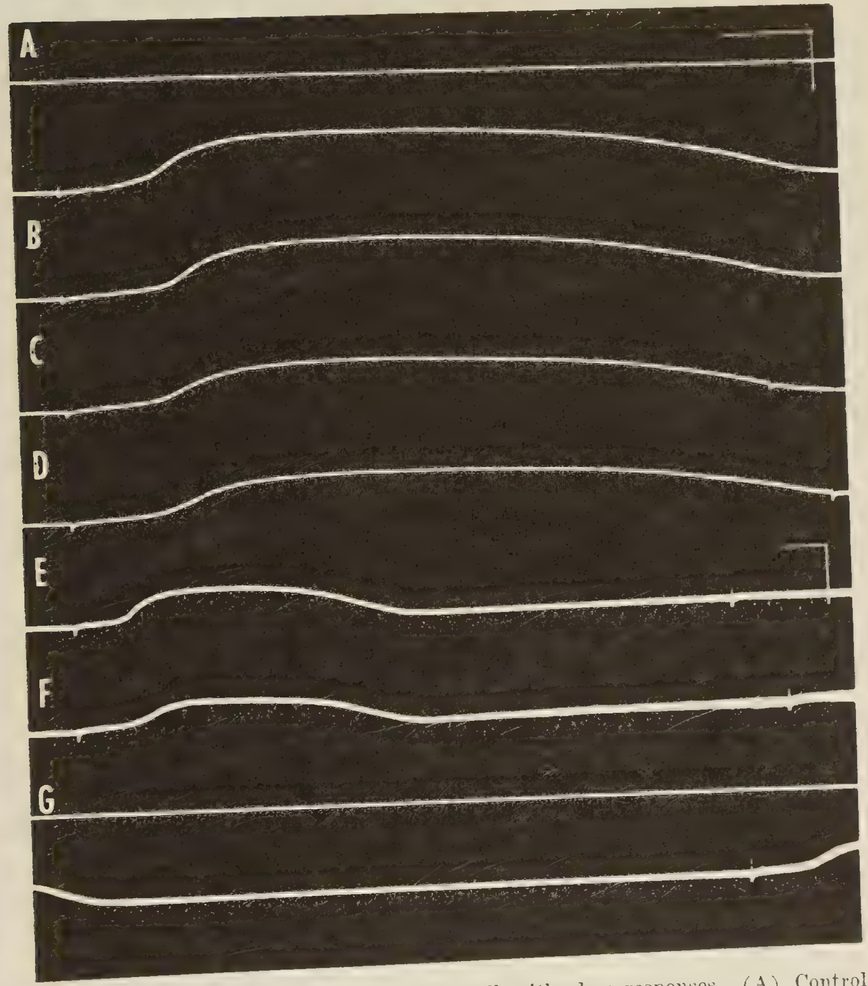

Fig. 11 Recovery period of a muscle cell with slow responses. (A) Control response. Interval between paired stimuli in (B) 420 msec., (C) 1.5 sec., (D) 1.6 sec., (E) 2.8 sec., (F) 3.01 sec. and (G) 4.2 sec. First lines in (A) and (G) represent zero potential. Vertical bar represents 50 mv; horizontal bar, 200 msec. Note when (G) was recorded, resting potential had decreased from —93 mv to —68 mv.

gested that a differentiation of function of skeletal muscles may exist as early as the 7th day *in vitro.*

3. *Resting membrane potentials.* In this study resting potentials from approximately 500 muscle fibers were recorded. However, the range of these measurements was so wide that little confidence could be placed in their true values. The results presented below (table 1) were chosen from 35 fibers which showed (1) either spontaneous spike discharges or responses to electrical stimulation and (2) that the recorded zero potential before the penetration was in agreement with that obtained after the withdrawal of the microelectrode. These measurements indicate that there was no significant difference in the values recorded from fibers with twitch or with slow responses.

TABLE 1

Resting membrane potentials of cultured chick skeletal muscle cells

	FIBERS SHOWING TWITCH RESPONSES OR SPONTANEOUS DISCHARGES	FIBERS SHOWING SLOW RESPONSES	ALL FIBERS
No. of fibers	17	18	35
Resting potentials (— mv)			
Range	40–93	48–90	40–93
Average	67 ± 4 (S.D.)	64 ± 8 (S.D.)	66 ± 5 (S.D.)

DISCUSSION

The remarkable resemblance between the spontaneous rhythmic spike potentials from chick embryonic skeletal muscle grown in tissue culture and the fibrillation potentials from denervated mammalian skeletal muscle suggests that the mechanisms for the production of these potentials are perhaps basically the same. The explanted muscle *in vitro* gives rise to new growth of muscle cells which mature and show all the histological features of normal adult muscle (fig. 2B) with two exceptions: (a) the diameter of the fibers being 15 μ, comparable with that of denervated adult muscle; (b) a search for neuronal elements or motor end-plate organs having been repeatedly fruitless, both in living cultured tissue by means of phase contrast microscopy and in fixed preparations by the Janus Green method of Couteaux ('47) and Coers' modification ('55) of the Koelle method for cholinesterase (Hasse and Engel, '58). The origin of these spontaneous rhythmic potentials must, therefore, be sought in the muscle fiber other than at the end-plate region.

Spontaneous rhythmic oscillations of potential have also been found in neurones of the cerebral cortex, especially when the neurones were poisoned by strychnine (Li, '59). In other types of nerve elements an accentuation of oscillating potential was produced by sustained depolarization (Arvanitaki, '40 and Cole, '49), or a change in the external environment (Brink, Bronk and Larabee, '46; Shanes, '49 and Adelman, '56). Under these circumstances the amplitude of the oscillating potentials was apt to increase sufficiently to initiate successive spike discharges (Shanes, '58). In the present study, when spontaneous spike discharges failed to occur, spontaneous rhythmic oscillations of potentials still continued with precisely the same frequency. If there was a dissociation of spike discharges and oscillations, the spike discharges appeared to depend upon the critical level of depolarization which the oscillating potentials had achieved. The occurrence of spike potentials did not seem to interfere with the natural rhythm of the oscillating potentials.

Fibrillation potentials from mammalian skeletal muscle fibers *in vivo* were previously recorded with an intracellular microelectrode two days after denervation. The resting membrane potentials of these fibers might be absolutely stable for some time, but was generally fluctuating (Li et al., '57). It is known that muscle fibers undergo a tropic change after their motor nerve has been severed. A biochemical study of the rat diaphragm following neurotomy suggested a change in hexokinase phosphorylase, and cytochrome oxidase activity (Humoller, Hatch and McIntyre, '51 and '52). Other undisclosed metabolic changes may also occur.

On the basis of the present observations, a mechanism for the initiation of spontaneous rhythmic spike discharges of chick embryonic skeletal muscle *in vitro* is suggested. This mechanism may also apply to fibrillation potentials from denervated mammalian skeletal muscle fibers. The sequency of events may be as follows: (1) denervation, (2) change in muscle metabolism, (3) instability of resting membrane potential, (4) spontaneous rhythmic oscillations of membrane potential and (5) spontaneous rhythmic spike discharges.

SUMMARY

Skeletal muscle cells of the chick embryo were investigated with glass micropipette electrodes after growing 7–21 days in tissue culture. This study has led to the following results and conclusions:

1. Stimulation of the muscle evoked two types of responses, suggesting that a differentiation of function may exist in the embryonic muscle cells.
2. Cells with slow responses might remain inactive after excitation for as long as 4.2 seconds and take no part in the initiation of spontaneous rhythmic spike discharges.
3. Cells with twitch responses had a refractory period ranging between 25–35 msec. and were responsible for the spontaneous rhythmic spike discharges. The spike generated from these cells might be as large as 100 mv and 2.0 msec.
4. Resting membrane potentials showed no significant difference in cells with twitch responses and with slow responses, nor was there any change with age of the cells from 7 days to 21 days. Reliable measurements ran from —40 to —93 mv with an average of —66 ± 5 mv (S.D.).
5. Spontaneous rhythmic oscillations of potential could occur in the absence of spike discharges; but having attained a critical level of depolarization, they initiated spike discharges. The spike discharges did not interfere with the rhythm of the oscillating potentials.

6. Since the behavior of the spontaneous rhythmic spike discharges from cultured muscle cells and the fibrillation potentials from denervated mammalian skeletal muscle fibers were remarkably similar, it is proposed that the mechanism of the fibrillation in both may follow this sequence: (1) denervation, (2) change of muscle metabolism, (3) instability of resting membrane potential, (4) spontaneous rhythmic oscillations of membrane potential and (5) spontaneous rhythmic spike discharges.

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EXTRACTED MAMMALIAN SPERM MODELS

I. PREPARATION AND REACTIVATION WITH ADENOSINE TRIPHOSPHATE

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Developed by Szent-Györgyi ('49) and Varga ('50), the glycerine fiber bundle model has become a standard experimental system in the field of muscle biochemistry and physiology. Refinements and applications of the technique have led to the preparation of single muscle fiber models (Weber and Weber, '50), of extruded actomyosin thread models (Portzehl and Weber, '50) and of compressed monolayer actomyosin models (Hayashi, '52). All these systems respond promptly by contraction upon the addition of adenosine triphosphate (ATP) administered in the proper concentration and ionic environment. Maximal tensions exerted by the models have the same order of magnitude and temperature dependence as those of the corresponding living systems (Weber, '55).

Hoffmann-Berling ('54a) and more recently Alexandrov and Arronet ('56) have extended the cell model approach to a study of nonmuscular systems. Convincing evidence has now been provided which indicates that contractile protein is responsible, as it is in muscle, for movement in a variety of cells and organelles, such as the myoneme of the stalked *Vorticella* (Hoffmann-Berling, '58), the flagella of grasshopper spermatozoa (Hoffmann-Berling, '55), the cilia of the pharyngeal mucous membrane (Alexandrov and Arronet, '56),

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and the fibroblasts of rat sarcoma grown in tissue culture (Hoffmann-Berling, '54b). While the precise mechanism of contraction and relaxation may vary in these different systems, the presence of contractile protein as a basis for motility seems well established. Contractile protein has, moreover, been isolated and characterized from fibroblast cells (Hoffmann-Berling, '56) and a comparable functional protein has been extracted in crude form from bull testes (Bishop, '58a). The isolation of functional contractile protein from sperm tails would confirm the supposition of Engelhardt ('46) who postulated an actomyosin-like substance as the basis for sperm motility.

ATPase activity has been demonstrated in sperm (Lardy et al., '45) and in suspension of sperm tails (Nelson, '55). Engelhardt and Burnasheva ('57) report the isolation, from whole cells and from sperm tails (bull), of a protein fraction, "spermosin," which enzymatically splits ATP. Eighty percent of the ATPase activity of whole sperm homogenates is concentrated in the tail fraction. The enzyme is activated by Ca^{++}, is sensitive to sulfhydryl poisons (cadmium chloride and *p*-chloromercuribenzoate), and spilts ADP almost as effectively as ATP. These results tend to confirm those of Nelson ('54) who claimed high splitting activity in both KCl and water-extracted preparations of bull sperm flagella.

Hoffmann-Berling ('55) prepared glycerine-extracted cell models of sperm of the grasshopper, Tachycines, but the procedure proved unsuccessful with mammalian sperm. The present report concerns extraction and reactivation methods found applicable to the preparation of what we regard as true sperm models from 6 mammalian species. The characteristics of the reactivated cells and the conditions underlying the reactivation process are treated in a qualitative manner, the basic criterion for reactivation being the occurrence of ATP-induced rhythmic flagellation in the extracted cells. A more quantitative analysis of the rate and conditions of ATP-splitting activity and the relative "tensions" developed by reactivated sperm will be published in subsequent papers.

MATERIALS

The sources of spermatozoa used in this investigation included the rabbit, guinea pig, rat, mouse, bull and man. Seminal sperm, obtained by ejaculation of the rabbit and man, were resuspended in Tyrode's solution before extraction. Epididymal sperm were collected immediately after sacrifice from the rabbit, guinea pig, rat, mouse and bull and were also diluted in Tyrode's solution. An excellent source of material proved to be bull sperm, back-flushed from the epididymis by Tyrode's solution gently forced into the vas deferens by means of a glass syringe (5 ml) and 20-gauge needle. Such spermatozoa, uncontaminated by seminal plasma, are highly motile and may be conveniently refrigerated for 6 to 8 hours; the excised epididymides may be stored overnight at about 2°C before expressing the sperm in a highly viable condition. However, an advantage is to be gained in the use of fresh material since the higher the rate of motility before extraction, the more successful is the reactivation process.

METHODS

Extraction of sperm

Preliminary experiments indicated that all steps in the procedure can be carried out at room temperature (21–23°C). Subsequently, it was found that better reactivation occurs when extraction is performed in the cold (1–3°C). The standard technique now adopted involves cooling the sperm suspension during one hour to about 2°C before adding extraction medium, previously chilled to the same temperature. After extraction, the preparation is brought to room temperature for reactivation. If diluted out to 50% with glycerine, the extracted sperm may be stored at –20°C for periods up to three months or more, prior to reactivation with ATP (Bishop, '58b).

The basic extraction medium, applicable to all species of mammalian sperm studied, is shown in table 1. Both EDTA (disodium ethylenediaminetetraacetate) and digitonin are essential for effective extraction. Polyvinylpyrrolidone (PVP) is required, possibly serving both as a viscous support and as a colloid-osmotic agent. It may be replaced by chick serum or by 1% agar but not, surprisingly enough, by dextran. KCl may be replaced by NaCl, but not by KI. Magnesium ion, necessary for ultimate reactivation, is maintained in excess. Calcium, on the other hand, must be removed during extraction, and this suggests a role played by the chelating agent, EDTA.

TABLE 1

Extraction and reactivation media for mammalian spermatozoa

COMPONENT	EXTRACTION MEDIUM	REACTIVATION MEDIUM
KCl	0.12 M	0.16 M
KH_2PO_4 K_2HPO_4 [1]	0.01 M	0.01 M
EDTA [2]	0.005 M	—
$MgCl_2$	0.006 M	0.004 M
$CaCl_2$	—	0.001 M
Thioglycollate	0.001 M	—
Digitonin	0.01%	—
PVP [3]	3.5%	3.5%
ATP [4]	—	0.001 M
pH	7.0	7.0
Ionic strength	0.18 μ	0.21 μ

[1] Primary and secondary salts in proportion to yield proper pH.

[2] Disodium ethylenediaminetetraacetate (Versene).

[3] Polyvinylpyrrolidone, av. mol. wt. 30,000.

[4] Adenosine triphosphate, crystalline disodium salt, Pabst Laboratories.

The relative volume of sperm suspension to extraction fluid generally used is 1:4 or 1:9, depending on the density of the sperm suspension. Since the rate of extraction and loss of motility are directly dependent on the effective concentration of digitonin, this reagent must be controlled within reasonably narrow limits. Digitonin, a β-glycoside and steroid detergent, can selectively sequester certain bonds and combine stoichiometrically with derived lipids such as cholesterol, the higher alcohols, phenols and thiophenols. Extraction does not occur with insufficient digitonin; on the other hand, too high a concentration irreversibly destroys the capacity for motility.

Under optimal conditions sperm motility is stopped promptly upon the addition of extraction fluid — in bull sperm, for example, within 10 seconds. Rapid extraction seems to favor subsequent reactivation. During the extraction process all soluble organic and inorganic substances are presumably removed, permeability is destroyed and the normal ionic balance is upset. The extracted cells display neither fructolytic activity nor oxygen consumption. Morphologically, however, they appear quite normal when viewed in the phase contrast microscope.

While the procedure noted above has thus far proved most satisfactory for the various species of mammalian sperm investigated, modifications in the extraction medium permit reactivation, but of less successful sorts. Bull sperm respond to ATP after extraction without EDTA, but the motility displayed is feeble and of short duration. They can indeed be extracted simply in 50% glycerine (2°C), but the response to ATP is slow and the resulting motility is weak, involves only a fraction of the sperm treated, and lasts for only a brief period (15 to 30 minutes). In comparison, EDTA-digitonin extraction is much more satisfactory, providing models which quickly respond to ATP by vigorous and persistent flagellation.

Reactivation of extracted sperm

Reactivation as used here refers to the resumption and persistence of rhythmic motility upon the addition of ATP. The standard reactivation solution, satisfactory for all these species of mammalian sperm, is indicated in table 1. Mg^{++} is essential, and Ca^{++}, while apparently not required, improves the vigor of flagellation. PVP is required for extended rhythmicity. EDTA and digitonin inhibit reactivation and are of course omitted. The total ionic strength ($0.21\ \mu$) is somewhat higher than that of the extraction medium. Pyrophosphate (5×10^{-3} M) improves the amplitude of induced flagellation of rodent sperm but shows no effect on bull sperm. Although this medium (table 1) has been found most satisfactory for continued flagellation for mammalian sperm, the importance of ATP, and ATP alone, in inducing motility in the extracted cells is demonstrated by the fact that the nucleotide can be administered, with positive results, simply in Tyrode's solution or in buffered isotonic KCl or NaCl containing Mg ions.

The process of reactivation has, for the most part, been observed by direct microscopic observation of the spermatozoa during treatment. A glass slide, supporting an elevated cover slip, provides a chamber which is open at both ends and permits rapid replacement or renewal of the medium surrounding the cells. A single sperm can be observed and photographed throughout the entire procedure by trapping it in extraction medium made up in 1% agar. Mass reactivation, however, is accomplished simply by dilution, or by slow centrifugation and resuspension, of extracted sperm in adequate volumes of reactivation solution.

RESULTS

Nature of the induced motility

Time required for reactivation. Activity is resumed almost immediately upon the addition of ATP to a suspension of extracted bull sperm, but an appreciable time may lapse for sperm of other species (table 2). This apparent lag difference may be due to one or more of several factors: (1) the intrinsic biochemical conditions involved in the contraction-relaxation process vary in the different types of sperm; (2) the rates

TABLE 2

Time of initiation and duration of ATP-induced motility of mammalian sperm
(ATP, 10^{-3} M; $MgCl_2$, 4×10^{-3} M; temp., 22°C)

SPECIES	MINIMAL REACTIVATION TIME	APPROXIMATE DURATION OF MOTILITY
	seconds	*minutes*
Bull	~ 2	140
Mouse	14	30
Rat	15	60
Man	55	15

of diffusion, that is, the efflux of residual extracting substances (e.g., EDTA) or the influx of reactivating agents (e.g., ATP, Mg^{++}), differ from sperm to sperm; (3) the effect of ATP as a plasticizing agent differs in sperm of different species, thereby more rapidly rendering some sperm more flexible and capable of motility than others. That the third alternative is at least partly apt is indicated in the case of rodent sperm in which the latency of reactivation is considerably reduced by the addition of pyrophosphate (5×10^{-3} M), a plasticizing substance, at a constant concentration of ATP (5×10^{-4} M). Pyrophosphate, however, has no beneficial effect on the reactivation process of bull sperm, which respond quickly and maximally over the entire effective concentration range of ATP.

Duration of induced motility. At room temperature, rhythmic wave motion, induced by ATP, may last from several minutes, and involve relatively few beats, to two or more hours of vigorous and regular movement (table 2). Considerable species difference is shown and roughly parallels the relative survival capacity of fresh sperm under comparable conditions *in vitro.* The models are highly sensitive to temperature changes. An increase from 20 to 26°C impairs the duration of induced motility of bull sperm; at 30°C reactivation does not occur.

The rhythmic flagellation. Unlike muscle fiber models which contract but once upon the addition of ATP, sperm models assume a rhythmic motility which frequently looks not unlike that of normal sperm. The flagellation may be as vigorous or even may exceed that of the corresponding living cells. The amplitude of vibration varies but may approximate that of the normal wave vibration — in bull sperm, for example, 7–8 μ. Wave frequency varies from a slow beat to a rapid oscillation greater than that of normal sperm, e.g., in bull sperm about 9/sec. The entire tail may be involved or only relatively short segments of it, located either proximally or distally along the flagellum. Bending waves develop out from both sides of the longitudinal axis, much as they do in normal sperm.

In three important respects, however, the motility of reactivated sperm models is strikingly different from that of fresh cells. Rarely, if ever, do they undergo any pronounced forward progression. Occasionally one will be found which moves forward slowly and jerkily, but just as often another will be observed to move in a reverse direction. Moreover, in none of the reactivated sperm is rhythmic wave formation accompanied by either wave propagation along the flagellum or by cell rotation along the longitudinal axis, both characteristic of normal sperm movement (Gray, '55, '58). These failures indicate that the properties of wave co-ordination and propagation are lost during the extraction process (Bishop, '58d). Repeated attempts to restore these capacities by adjustment of the ionic balance were quite unsuccessful.

Reactivated sperm are capable of exerting some force, as determined by varying the viscosity of the medium in which they are bathed. Preliminary studies with methylcellulose (Tylose) solutions indicated that ATP-induced motility of extracted bull sperm can occur in media of about one-half the relative viscosity of solutions which just prevent motility of living cells. While this may not represent a measure of the true "tension" exerted by these cells, it does confirm the hypothesis that work is being done and energy expended by the reactivated cells. The validity of the method of viscous suppression of flagellation is supported by the analyses of Taylor ('52) and Hancock ('53), who showed that normal motility of sperm is related to viscous rather than to inertial forces of the medium.

Isolated sperm tails. Sperm tails, isolated from the heads, can be reactivated along with the intact cells. In an attempt to produce large numbers of isolated tails and tail fragments, extracted bull sperm in 50% glycerine were ground with sand after freezing at —20°C and then warmed to room temperature for reactivation with ATP. In such preparations motility is induced in entire tails and in those with at least some if not most of the mid-piece. Short sections of flagella do not respond to ATP. While this technique is not without its drawbacks, it would appear that the basal corpuscle or a considerable portion of the sperm mid-piece is required for motility of the flagellum.

Factors affecting induced motility

Nucleotide dependence. Motility is induced in extracted mammalian sperm by ATP over a range of 7×10^{-5} to 9×10^{-3} M. At 10^{-2} M and higher, the concentration of ATP is supraoptimal and motility fails to occur. In none of the species of mammalian sperm is there a clear indication of a correlation between ATP concentration and frequency or amplitude of beat. The response of extracted bull and rabbit sperm over the entire range of effective ATP concentrations is essentially flat; occasionally a high frequency is displayed at low concentration (10^{-4} M) and a slow rate of beat at high levels (5×10^{-3} M). Only in the rodent sperm which normally, i.e., in living cells, show a slower and less violent flagellation than that of bull or rabbit sperm, is there a suggestion that motility of the reactivated cells is ATP-limited at low concentration. Since, however, this weak response at low concentration of ATP can be boosted by the addition of pyrophosphate (5×10^{-3} M), the difference in response to ATP is probably due to its plasticizing effect rather than to its energizing activity. A similar relation was found by Hoffmann-Berling ('55) in grasshopper sperm and trypanosome models.

Motility can be induced in extracted bull sperm by adenosine diphosphate (ADP) in concentrations from 4×10^{-4} to 6×10^{-3} M. Inosine triphosphate (ITP) induces motility, albeit not very vigorous, at a concentration of 10^{-3} M, the only concentration tested. An increase in Mg^{++} concentration from 4×10^{-3} to 10^{-2} M has no effect on ITP-induced motility.

In the absence of any nucleotide, motility fails to occur in these extracted cells. Pyrophosphate alone, or in combination with other inorganic ions, is ineffective in the restoration of movement.

The reactivated sperm, resuspended in Tyrode's solution or in histidine- or tris-maleate-buffered media, can split ATP and this reaction is poisoned by such sulfhydryl reagents as Salyrgan.[2] The details and conditions of these reactions will be published elsewhere.

Ionic requirements. The reactivation process is magnesium-dependent with an optimum at 4 to 5×10^{-3} M. Under the conditions of the extraction, however, in which there is an excess of Mg^{++}, it is difficult to state precisely what are the limits of the effective concentration. In addition to the bound Mg^{++} which may be present after any extraction (Spicer and Bowen, '51), the free Mg^{++} as a carry-over from the extraction medium accounts for the reactivation of bull sperm by ATP added in buffered isotonic KCl or NaCl (table 6). The concentration of Mg^{++} in the *extraction* medium containing EDTA

TABLE 3

Effect of magnesium concentration in the extraction and reactivation media on the initiation of ATP-induced motility

MG^{++} CONCENTRATION, MOLAR	TIME OF REACTIVATION
	seconds
In extraction medium [1,2]	
6×10^{-3}	< 2–10
4×10^{-3}	15–30
2×10^{-3}	45–70
No Mg^{++} added	56–188
In reactivation medium [3]	
4×10^{-3}	15–30
2×10^{-3}	> 110

[1] Extraction medium includes EDTA, 5×10^{-3} M; digitonin, 0.01%.

[2] Reactivation medium (upper series) includes $MgCl_2$, 4×10^{-3} M.

[3] Extraction medium included EDTA, 5×10^{-3} M; digitonin, 0.01%, $MgCl_2$, 4×10^{-3} M.

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[2] Salyrgan (mersalyl): Salicyl-hydroxymercuri-methoxypropyl-amidoorthoacetate. The Salyrgan used in this investigation was supplied through the courtesy of the Sterling-Winthrop Research Institute, Rensselaer, New York.

definitely affects the rate of response when ATP (10^{-3} M) and Mg^{++} (4×10^{-3} M) are held constant in the reactivation medium (table 3). Reactivation is rapid when the sperm have been extracted in an excess of magnesium; it is slow when magnesium has been deficient. Moreover, the final concentration of Mg^{++} in the *reactivation* medium also affects the rate of response (table 3). ATP-induced motility occurs within 15 to 30 seconds at a magnesium concentration of 4×10^{-3} M; movement is restored only after about two minutes or more when the magnesium is reduced to 2×10^{-3} M or less.

Calcium is not essential, since it can be omitted from the reactivating medium, but its presence definitely improves the quality of flagellation. Both the amplitude and frequency of beat of bull sperm models tend to decrease when the Ca^{++} concentration is reduced from 10^{-3} to 10^{-4} M. Unlike the effect of Mg^{++}, changes in Ca^{++} have no effect on the time of reactivation. Ca^{++} cannot replace Mg^{++} in the activation of the models by ATP.

TABLE 4

Effect of Marsh-Bendall factor on the response of extracted bull sperm to ATP
(ATP, 10^{-3} M; $MgCl_2$, 4×10^{-3} M; $CaCl_2$, 2×10^{-3} M)

TREATMENT OF EXTRACTED CELLS	REACTIVATION RESPONSE TO ATP
Sperm + rabbit serum	+
Sperm + Marsh-Bendall factor + Ca^{++}	+
Sperm + Marsh-Bendall factor − Ca^{++}	−
Ca^{++} added to above preparation	+

Marsh-Bendall factor extracted from rabbit skeletal muscle according to the procedure of Bendall ('53).

With the further thought that calcium might have another type of action, namely by its effect on a relaxing system in the sperm, tests were carried out with Marsh-Bendall factor isolated from rabbit skeletal muscle according to the procedure of Bendall ('53). Extracted bull sperm mixed with active factor for 5 minutes failed to respond to ATP (10^{-3} M); after 15 minutes, however, the addition of Ca^{++} immediately restored motility (table 4). Control preparations of extracted sperm treated with Marsh-Bendall factor, inhibited by Ca^{++}, responded promptly upon the addition of ATP.

Since potassium can be replaced by sodium in both extraction and reactivation media, K^+ is not regarded as playing any ion-specific role in the reaction. Extraction in solutions made up predominantly of either univalent cation would be expected to produce serious changes in both permeability and ionic balance. While reactivation occurs best in solutions of a total ionic strength (0.21 μ) higher than that of physiological conditions (~0.18 μ), ATP-induced motility can also occur at 0.16 μ and perhaps even lower ionic strengths.

The pH of the reactivation medium may be varied over quite a wide range without blocking the reaction. The optimal hydrogen-ion concentration, which permits longest survival of all 6 types of mammalian sperm, is between pH 7.0 and 7.2. However, this may be extended from 6.85 to 7.40 and motility is still restored. While no parallel need be drawn between the pH tolerance of live and of extracted cells, it is of interest that most fresh mammalian sperm remain motile throughout a pH range of about 6.0 to 8.5.

Glycerine extraction. Extraction in 50% glycerine is the standard procedure for the preparation of muscle fiber models. Mammalian spermatozoa, however, are only poorly extracted in glycerine. If extracted, then their subsequent response to ATP is weak and sporadic.

Treatment in 50% glycerine has, however, been adopted here with two aims in view. The first is an academic one, namely to get the extracted sperm cells in an environment as near as possible like that of the muscle fiber models, as a basis for comparison. The second reason is the technical one of storage. Extracted sperm can be maintained at —20°C for long periods — at least three months in the case of bull sperm — but only if diluted out to 50% with glycerine. These preparations can be made in either of two ways: (1) After extraction of the sperm in KCl solution at 1 to 3°C, the entire suspension is diluted with cold glycerine (1:1). (2) The extraction medium is made up in 50% glycerine and this is used for extraction of the sperm in the cold. The beneficial effects of glycerine on stored, extracted bull sperm are indicated in table 5. Since the basic extraction medium contains digitonin as an essential component, and since without it glycerine is ineffectual in causing successful extraction, the term, "glycerine extraction," seems here to be a misnomer. Nevertheless, glycerine may play some ancillary role during the process of extraction, in the presence of digitonin, besides serving to protect the extracted cells during storage.

TABLE 5

Duration of reactivation capacity of extracted bull sperm stored with and without 50% glycerine

STORAGE MEDIUM	SURVIVAL, DAYS 2°C	SURVIVAL, DAYS −20°C
Extraction medium	4	3
Glycerine plus extraction medium	13	> 90

TABLE 6

Summary of responses to ATP of extracted bull spermatozoa
(ATP, 10^{-3} M; temp., 22°C)

CONDITIONS FOR REACTIVATION	ATP-INDUCED MOTILITY
Std. reactivation medium	+
Tyrode's solution	+
KCl, 0.16 M	+
NaCl, 0.16 M	+
KI, 0.16 M	−
Marsh-Bendall factor without Ca^{++}	−
Marsh-Bendall factor with Ca^{++}	+
Salyrgan, 5×10^{-4} M	−
Salyrgan and cysteine, 10^{-3} M	+
Trypsin, 0.01%, 15 min.	−
Papain, 0.02%, 30 min.	−
Sonic vibration, 30 sec.	−
Homogenation, 60 sec.	−
Exposure to 30°C, 30 min.	−

Inhibitors and deleterious agents. Motility is completely inhibited by the sulfhydryl reagent, Salyrgan, at a concentration of 5×10^{-4} M, when mixed with the cell suspension before the addition of ATP. If Salyrgan and ATP are added simultaneously, bull sperm models respond, are motile for one to two minutes, and then cease movement. The inhibition by Salyrgan is partially counteracted by 10^{-3} M cysteine (table 6).

Extracted sperm (bull and rat) are sensitive to the proteolytic enzymes, trypsin and papain (table 6). A 15-minute exposure to trypsin (0.01%) or a 30-minute treatment with papain (0.02%) prevents subsequent reactivation. Cells treated in this way show little if any morphological change; longer exposures to either of these enzymes lead to proteolysis of the sperm tail. The initial enzymatic damage probably involves the contractile protein, but injury could also be incurred by structural components necessary for wave formation.

Either homogenation in the blender for 60 seconds or sonic vibration at 8900 cps for 30 seconds causes irreversible changes in extracted sperm which prevent their reactivation with ATP (table 6). Presumably these deleterious effects are associated with denaturation of contractile protein.

DISCUSSION

From the evidence in literature previously cited and conclusions reached in other investigations concerning the enzymatic (Nelson, '58) and ultrastructural (Astbury and Weibull, '49; Afzelius, '59) nature of flagella, there is little doubt that contractile protein constitutes the physical basis for motility in spermatozoa. The behavior of sperm, extracted in KCl media or in KCl-glycerine solutions and subsequently reactivated by ATP in a suitable concentration and ionic milieu, supports this contention. The cell model, or reconstituted cell, concept as described here seems to apply as well to mammalian spermatozoa as to muscle fiber preparations. The crux of the problem, of course, rests on the eventual extraction and identification of the contractile proteins as they exist in sperm flagella.

The initiation and rhythmicity of contraction-relaxation cycles induced in sperm models by ATP are naturally a source of bewilderment. At the same time, it is unreasonable to expect flagella to function exactly as do muscle models which contract only once upon the addition of ATP, so long as the external conditions remain the same. Whether sperm motility is envisaged as due to fibril shortening (Bradfield, '55) or as a sliding of adjacent filaments one upon the other (Afzelius, '59), each "contraction wave" must be matched by a "relaxation wave" on the opposite side of the flagellum. Moreover, if the contraction wave is to be propagated, it must be preceded and followed by propagated waves of relaxation. Briefly put, part of the flagellum is always in a state of contraction and part of it is always undergoing relaxation. Theoretically, ATP could function in either the contraction or in the relaxation phase of the cycle. There is no evidence to suggest that either the entire longitudinal fibrils of the tail contract at once or that a spiral wave is initiated at the base and is propagated distally. The intrinsic synchrony between contraction and relaxation, characteristic of sperm motility, makes this movement all the more interesting and worth the study; it does not rule sperm out as potential cell models. The ultimate basis for this rhythmic activity depends on the precise arrangement of contractile proteins within the flagellum, the site and nature of the interreactions between ATP and contractile protein, the exact role of Mg^{++}, Ca^{++}, plasticizing and possibly relaxing factors in the fibrils, and perhaps to other features — about which we now know, unfortunately, even less for the process of sperm motility than we do for muscle contraction.

The characteristics of the induced flagellation demonstrate that this is due to active changes in the reactivated cells and not to some outside physical force, such, for example, as Brownian movement. Both the amplitude and the frequency of beat vary, to be sure, but they often equal or exceed that of living sperm. Wave amplitude in the models can attain a maximum of 7 to 8 μ. Moreover, the low rate of vibration sometimes observed is hardly consistent with movement resulting from Brownian action. If this force were of any consequence here, one would expect small tail fragments to show motility similar to that of intact tails and flagella of entire sperm. This is clearly not the case. The suggestion that ATP merely exerts a plasticizing influence on the flagella, thereby rendering them more flexible and able to respond to Brownian-type forces, is highly unlikely. ATP is known to retain its plasticizing capacity in muscle systems when both contraction and splitting activity are inhibited by either an increase in ATP to supraoptimal levels or by inhibition with Salyrgan (Weber and Portzehl, '54). Both types of inhibition occur in sperm flagellar models and yet motility is not restored merely as a result of the plasticizing action of ATP. Finally, the fact that the models can move despite an appreciable viscous resistance tends to eliminate such factors as Brownian movement, convection currents, and the like, as serious contenders for the force responsible for the motility observed in reactivated sperm.

The methods of extraction and reactivation evolved here for sperm are somewhat different from those found satisfactory for muscle fiber systems, but this in itself cannot be regarded as a serious objection to considering both as models. The most significant modifications in technique, the use of digitonin and EDTA, merely facilitate the extraction process and may be required only because of the tough fibrous sheath around the mammalian sperm and the advantage of binding calcium and possibly other ions during the critical process. Glycerine treatment of sperm may indeed be so gentle as to "preserve" rather than to extract the cells. Experience indicates that the extraction-reactivation procedures which apply to one type of sperm do not necessarily work with another. The technique used for grasshopper sperm (Hoffmann-Berling, '55) does not work on mammalian sperm, and the methods described here are not satisfactory for marine sperm such as those of the squid (Bishop, '58c). The physicochemical structure of the entire flagellum defines the particular procedure to be followed and this must be adapted to the type and species of cell.

While inadequate for extraction, glycerine proved to be essential as a storage medium, possibly serving to "stabilize" the contractile proteins. The interesting point arises that perhaps the addition of glycerine to these preparations merely preserves moribund cells, just as live sperm can be stored at extremely low temperatures, e.g., —79 or —196°C (Polge, '53). A series of tests with glycerine solutions of various concentrations, however, demonstrated that this is not the case. Extracted bull sperm models are preserved at —20°C only if the glycerine concentration is higher than 40%. Live sperm, on the other hand, are normally preserved in media with 10 to 18% glycerine and are destroyed when the concentration exceeds 25%.

The concentrations of ATP which induce motility in the sperm models approximate those of the physiological range. The upper limit (9×10^{-3} M) is of the same order of magnitude as the limiting ATP concentration for isolated muscle systems (Weber and Portzehl, '54). The negative responses at supraoptimal concentrations of ATP are possibly due to substrate inhibition. The positive responses recorded here for the action of ADP on cell models may be a direct effect, or may depend on its phosphorylation first to ATP. Presumably an enzyme with myokinase activity, responsible for the conversion, could be retained in the cells during the extraction process.

Magnesium must be regarded as an essential ion for the reactivation process with ATP, ADP or ITP. No significant differences in the concentration requirements of Mg^{++} by the three nucleotides were noted, but it is probable that such differences exist and might be discerned with a more quantitatively precise end point than the occurrence of degree of induced motility. ATP-splitting determinations do, in fact, indicate such differences in Mg^{++} requirements. The differences in the times of ATP reactivation, dependent on the concentration of Mg^{++} in the extraction or reactivation medium, further indicate subtle effects of this ion. These differences in reactivation times are not due simply to variations in diffusion rates of Mg^{++} or to variations in the rapidity with which extraction medium may be replaced by reactivating solution. They do reflect the amount of Mg^{++} available for activation processes involving ATP. Moreover, if there should prove to be a relaxing factor system operative in sperm, comparable to that of muscle, Mg^{++} might well be essential for this mechanism as well.

The role of calcium is decidedly unclear. Not an essential ion for the reactivation process, nevertheless it can play a significant part, at least under certain experimental conditions. By virtue of its combination with ATP, a chelating agent, under some circumstances Ca^{++} can reduce the effective amount of the nucleotide from supraoptimal to active working concentrations. The responses of bull sperm models to active and inactive Marsh-Bendall factor, and the significance of Ca^{++} in this reaction, suggest that spermatozoa may be equipped with their own relaxing system which is also sensitive to free calcium ions. While provocative, it is certainly premature to speculate that the continuing contraction-relaxation cycles of flagellation are in some way dependent upon the shift in the two cations, Ca^{++} and Mg^{++}, back and forth between the contraction and the relaxation systems. A somewhat different contraction-relaxation cycle, one involving Ca^{++} during contraction and ATP during relaxation, has been demonstrated by Hoffmann-Berling ('58, '59) in the myoneme of the stalk of *Vorticella.*

The interchangeability of K^+ and Na^+ in the extraction and reactivation procedure, without apparently affecting activity, has been demonstrated. This comes as no surprise, however, since the same kind of exchange of indifferent ions has little effect on either tension or shortening in the simplified muscle system (Weber and Portzehl, '54). The importance of K^+ in the intact muscle involves the processes of excitation and activation and not the fundamental process of contraction. It may well be that the failure of sperm models to undergo wave propagation and wave co-ordination, which normally result in spiral movement, is due to the complete upset in ionic balance in the extracted cells.

The extreme sensitivity of the models to a variety of agents which, at these dosages, are harmless to living cells indicates that the contractile proteins are easily and irreversibly denatured when exposed by extraction. The rapid action of proteolytic enzymes, sonic vibration and blenderization may involve some changes in structural protein, but the effects are more likely on the contractile substance itself. Similarly, the damage provoked by an increase in temperature from 20 to 30°C is also inherent in protein denaturation. This may be accompanied by physical deterioration such as that demonstrated by Matoltsy ('50) in glycerinated muscle fibers at temperatures above 20°C.

The fact that motility in the reactivated sperm is restricted essentially to self-perpetuating bending waves and displays neither wave propagation nor co-ordination is, in a sense, a source of gratification. It is highly unlikely that digitonin-treated, extracted cells would retain sufficient integrity to permit normal activity, involving as it must certainly do an integrated system with functional irritability, permeability and ionic balance. Sperm models are not normal cells and they do not move exactly as do living cells. Their induced rhythmic motility, however, does represent the activity of a functional contraction-relaxation mechanism.

SUMMARY

Methods have been described for the extraction and reactivation of sperm from 6 species of mammals: rabbit, guinea pig, rat, mouse, bull and man. The characteristics of ATP-induced motility and the conditions underlying the reactivation process justify the acknowledgment of these cells as true sperm model systems. The models are characterized by the following features:

1. Motility is induced in the extracted cells by ATP, ADP or ITP, but not by pyrophosphate or other inorganic ions.

2. Reactivation is magnesium-dependent and is sensitive to Ca^{++} under certain conditions.

3. The reaction is inhibited by the sulfhydryl reagent, Salyrgan, or by exposure to trypsin, papain, sonic vibration, blenderization, or temperature elevation to 30°C. All these agents inhibit ATP-splitting by the models.

4. Extracted bull sperm, stored in 50% glycerine at —20°C, maintain their capacity for reactivation for periods up to three months.

5. Motility is induced in bull sperm models within two seconds and may last two to three hours. A species difference exists in the time of initiation and duration of induced motility.

6. The induced motility is a rhythmic vibration with frequency and amplitude often, but not necessarily, like those of normal sperm. The entire tail may be active or only limited regions of it. Isolated flagella can be reactivated. No correlation exists between ATP concentration and frequency or amplitude of beat.

7. In contrast to the movement of normal sperm, the induced motility of the models displays no forward progression, wave propagation or spiral rotation.

The reactivated sperm models provide a tool for further study of the contraction-relaxation cycle in these specialized flagellated cells.

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